

IMMUNOREACTIVE CHANGES IN THE HYPOGLOSSAL  
NUCLEUS AFTER NERVE INJURY

1991

CURTIS



UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES  
F. EDWARD HÉBERT SCHOOL OF MEDICINE  
4301 JONES BRIDGE ROAD  
BETHESDA, MARYLAND 20814-4799



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Name of Candidate: Maria Curtis  
Master of Science  
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Thesis and Abstract Approved:

Committee Chairperson

7/24/91

Date

Committee Member

7/25/91

Date

Committee Member

7/25/91

Date





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Maria Curtis  
Department of Anatomy  
Uniformed Services University  
of the Health Sciences



ABSTRACT

Title of Thesis: Immunoreactive Changes in the Hypoglossal  
Nucleus After Nerve Injury

Maria Curtis, Master of Science, 1991

Thesis directed by: Dr. Rosemary C. Borke, Associate  
Professor of Anatomy, Department of  
Anatomy, USUHS

Choline acetyltransferase (ChAT) and calcitonin gene-related peptide (CGRP) are synthesized in the hypoglossal motoneuron. This study examines changes in ChAT and CGRP immunoreactivity (IR) after three types of hypoglossal nerve injury (resection, transection and crush) at 1, 3, 7, 20 and 50 days postoperative (dpo). Maximal reduction of ChAT immunostaining occurred at 7 dpo, and by 50 dpo had returned to normal for all injuries. CGRP immunostaining increased maximally at 1-3 dpo and at 50 dpo was normal for nerve crush, yet decreased for transection and resection. Cellular levels of ChAT and CGRP respond in diametric fashion, temporally and in regard to type of injury. Correlative data of reinnervation of tongue musculature were supplied by HRP labeling of hypoglossal motoneurons after nerve injury. These data provide a more complete picture of the role that a neurotransmitter-related protein, ChAT, and a putative trophic neuropeptide, CGRP, may play in nerve regeneration.

IMMUNOREACTIVE CHANGES IN THE HYPOGLOSSAL NUCLEUS  
AFTER NERVE INJURY

by

Maria Curtis

Thesis submitted to the Faculty of the Department of Anatomy  
Graduate Program of the Uniformed Services University of  
the Health Sciences in partial fulfillment of the  
requirements for the degree of  
Master of Science 1991

**DEDICATION**

**To Ron Johnson, my husband**  
**and** **Joseph**  
**Hester Curtis, my mom**  
**for their unwavering faith and support.**

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## TABLE OF CONTENTS

	<u>Page</u>
I. INTRODUCTION .....	1
Nerve Regeneration .....	1
The Hypoglossal Nucleus as a Model for	
Peripheral Nerve Regeneration .....	1
Anatomic Response of Neurons to	
Axotomy .....	2
Metabolic Response of Neurons to	
Axotomy .....	3
Choline Acetyltransferase .....	4
Calcitonin Gene-Related Peptide .....	5
Rationale .....	7
II. MATERIALS AND METHODS .....	8
Animal Population .....	8
Animal Surgery .....	8
Immunocytochemistry .....	13
ChAT Immunocytochemistry .....	13
CGRP Immunocytochemistry .....	14
Immunocytochemistry Quantitation .....	14
Statistical Analysis .....	16
HRP Labeling .....	16
III. RESULTS .....	18
Choline Acetyltransferase	
Immunoreactivity .....	18

IV. DISCUSSION	Qualitative Observations .....	18
	Unoperated Animals .....	18
	XII Nuclei Contralateral to	
	Axotomy .....	18
	XII Nuclei Ipsilateral to	
	Axotomy .....	18
	Quantitative Immunocytochemistry ...	21
	Average Intensity in Injured vs.	
	Uninjured XII Nuclei .....	21
	Calcitonin Gene-Related Peptide	
	Immunoreactivity .....	34
	Qualitative Observations .....	34
	Unoperated Animals .....	34
	XII Nuclei Contralateral to	
	Axotomy .....	34
	XII Nuclei Ipsilateral to	
	Axotomy .....	34
	Quantitative Immunocytochemistry ...	41
	Average Intensity in Injured vs.	
	Uninjured XII Nuclei .....	41
	HRP Labeling of Hypoglossal Nuclei .....	46
	XII Nuclei Contralateral to	
	Axotomy .....	46
	XII Nuclei Ipsilateral to	
	Axotomy .....	52

IV. DISCUSSION .....	65
Response of ChAT to Hypoglossal Nerve	
Injury .....	66
Response of CGRP to Hypoglossal Nerve	
Injury .....	68
V. CONCLUSION .....	75
BIBLIOGRAPHY .....	76

## LIST OF TABLES

		<u>Page</u>
Table I.	Statistical Analysis of ChAT-IR: Summary of F-values and p-values.	33
Figure		
Table II.	Statistical analysis of CGRP-IR: Summary of F-values and p-values.	49

## LIST OF FIGURES

		<u>Page</u>
<b>Figure 1.</b>	Surgical field.	10
<b>Figure 2.</b>	Types of nerve injuries.	12
<b>Figure 3.</b>	Light micrograph of normal ChAT-IR.	20
<b>Figure 4.</b>	Light micrographs of ChAT-IR, 7 dpo.	23
<b>Figure 5.</b>	Light micrographs of ChAT-IR, 7 dpo, high power.	25
<b>Figure 6.</b>	Light micrographs of ChAT-IR, 20 dpo.	27
<b>Figure 7.</b>	Light micrographs of ChAT-IR, 50 dpo.	29
<b>Figure 8.</b>	Difference in average intensity of ChAT-IR between uninjured and injured hypoglossal nuclei as a function of survival time.	32
<b>Figure 9.</b>	Light micrograph of normal CGRP-IR.	36
<b>Figure 10.</b>	Light micrographs of CGRP-IR, 1-3 dpo.	38
<b>Figure 11.</b>	Light micrographs of CGRP-IR, 7 dpo.	40
<b>Figure 12.</b>	Light micrographs of CGRP-IR, 20 dpo.	43
<b>Figure 13.</b>	Light micrographs of CGRP-IR, 50 dpo.	45
<b>Figure 14.</b>	Difference in average intensity of CGRP-IR between uninjured and injured hypoglossal nuclei as a function of survival time.	48
<b>Figure 15.</b>	HRP labeling in hypoglossal neurons after axotomy.	51

	<u>Page</u>
<b>Figure 16.</b> Dark field micrograph of HRP labeling, 20 dpo nerve crush.	54
<b>Figure 17.</b> Dark field micrograph of HRP labeling, 20 dpo nerve transection.	56
<b>Figure 18.</b> Dark field micrograph of HRP labeling, 20 dpo nerve resection.	58
<b>Figure 19.</b> Dark field micrograph of HRP labeling, 50 dpo nerve crush.	60
<b>Figure 20.</b> Dark field micrograph of HRP labeling, 50 dpo nerve transection.	62
<b>Figure 21.</b> Dark field micrograph of HRP labeling, 50 dpo nerve resection.	64

## INTRODUCTION

### Nerve Regeneration

A fundamental problem in neurobiology is understanding the process of nerve regeneration. The consequences of nerve trauma, often devastatingly permanent, have sustained a great clinical interest in determining the neuron's regenerative capacity. The task of a mature neuron is communication; after injury, the formerly communicative neuron is isolated from its afferent source of information and peripheral target. When the axon is severed, the cell soma undergoes profound morphologic and metabolic changes. These changes shift the synthetic priority of the cell body to growth-associated products, and de-emphasize synthesis of neurotransmitter-related products (Lieberman, 1971). The elucidation of the cellular signals responsible for determining synthetic priorities could be of clinical benefit to the management of nerve injury cases.

### The Hypoglossal Nucleus as a Model for Peripheral Nerve Regeneration

It is known that peripheral nervous system (PNS) neurons possess a greater regenerative ability than central nervous system (CNS) neurons (Barron, 1983). In the rat, the hypoglossal (XII) nucleus is located in the medulla of the brainstem, and consists mainly of motoneurons (Cooper, 1981). The axons of these motoneurons combine to form the

hypoglossal (XII) nerve, which innervates the intrinsic and extrinsic musculature of the tongue. The nerve is accessible for surgical intervention along its route from the cranium to the tongue. Since the XII nucleus is a purely motor nucleus (Carpenter & Sutin, 1983), it provides a convenient paradigm to study peripheral motoneuron regeneration without interference of sensory fibers (Brattgard et al., 1957; Watson, 1968; Rotter et al., 1977; Wooten et al., 1978; Aldskogius et al., 1980; Lams et al., 1988; Armstrong et al., 1991).

#### Anatomic Response of Neurons to Axotomy

Nerve insult to the PNS consistently elicits a response called the retrograde or axon reaction. Variation in the reaction has been documented between different neuronal populations, types and locations of injury, and developmental states (Lieberman, 1974). First delineated by Nissl (1892), the classical response of the nerve cell body to axotomy is characterized by chromatolysis, a change in the rough endoplasmic reticulum (rER). Normal rER appears as concentrations of flat elongated cisternae arranged in parallel arrays that are externally lined with membrane-attached ribosomes and have free polyribosomes between adjacent cisternae. Chromatolysis is characterized by an irregularity in the organization of the cisternal arrays, a detachment of membrane associated ribosomes and an increase in free polyribosomes (Grafstein and McQuarrie, 1978).

Other consistent morphologic changes in the cell body include nuclear eccentricity, cell body swelling, and an increase in nucleolar size (Lieberman, 1971; Grafstein & McQuarrie, 1978).

#### Metabolic Response of Neurons to Axotomy

Nerve injury also provokes metabolic changes in the neuron. Increased nucleolar RNA and protein content, as well as increased RNA synthesis, occur in the hypoglossal nucleus subsequent to nerve insult (Watson, 1968). An increase in cytoplasmic protein synthesis and cell content of RNA has been observed, and remains elevated regardless of the neuron's eventual fate (Watson, 1968; Barron, 1983; Smith, 1984). A change in protein routing has been documented (Whitnall & Grafstein, 1982, 1983). Enzymatic and energy requirement changes have also been reported (Bodian & Mellors, 1945; Kreutzberg, 1963; Smith, 1984).

Nerve injury also elicits a metabolic switch toward de-emphasis of the synthesis of neurotransmitter-related products and re-emphasis of the synthesis of growth related products (Lieberman, 1971; Skene & Willard, 1981a,b).

Levels of synthetic enzymes for neurotransmitter biosynthesis have been shown to be reduced after nerve injury (Ross et al., 1979; Armstrong et al., 1991). Growth associated proteins (GAPs) have been found in neonatal animals and have been shown to decrease significantly with maturity (Skene & Willard, 1981b); GAPs have also been shown

to increase after axotomy (Skene & Willard, 1981a). In rat hypoglossal motoneurons, GAP-43 and GAP-23 have been shown to increase after XII nerve crush, to peak at 7 days postoperative (dpo), and to decrease upon reinnervation of tongue musculature (Redshaw & Bisby, 1984). Other proteins that have been shown to increase are tubulin and actin (Pearson et al., 1988), and an increase in glycosylation of proteins has been detected (Giulian et al., 1980). Shifts in the production of these proteins support the hypothesis of a change in synthetic priority subsequent to nerve injury.

#### Choline Acetyltransferase

Acetylcholine is the neurotransmitter of the hypoglossal motoneuron. Choline acetyltransferase (ChAT) is the biosynthetic enzyme of the acetylcholine production pathway (Tucek, 1990). ChAT has been localized immunocytochemically (Kimura et al., 1980; Armstrong et al., 1983; Houser et al., 1983) and ChAT mRNA has been localized by *in situ* hybridization (Cortes et al., 1990) in the hypoglossal nucleus.

Earlier studies that have examined changes in ChAT response to axotomy were chiefly biochemical in nature. A reversible loss in ChAT immunoassay was reported after hypoglossal nerve transection and resection (Gottesfeld & Fonnum, 1977; Wooten et al., 1978). Another study demonstrated a loss of muscarinic cholinergic receptors and

afferent synapses subsequent to XII nerve transection (Rotter et al., 1977). Recently, a decrease of ChAT immunoreactivity (ChAT-IR) has been documented in the hypoglossal nucleus after nerve crush and transection with ligation (Armstrong et al., 1991; Lams et al., 1988). Armstrong et al. (1991) demonstrated that nerve crush resulted in a smaller decrease in ChAT and was reversible in a shorter time period than in neurons whose axons were transected with ligation. These results are in agreement with the concept of the injured nerve cell shifting its metabolism from a neurotransmitter-oriented synthesis to the synthesis of growth-related proteins (Willard & Skene, 1981a,b; Redshaw and Bisby, 1984).

#### Calcitonin Gene-Related Peptide

Calcitonin gene-related peptide (CGRP) is a novel neuropeptide composed of 37 amino acids, alternatively processed from the calcitonin gene (Amara et al., 1982; Rosenfeld et al., 1983). CGRP has been shown to occur throughout the central and peripheral nervous system (Kawai et al., 1985; Ishida-Yamamoto & Tohyama, 1989; Rethelyi et al., 1989). It is found in cranial and spinal motoneurons and at the neuromuscular junction (Moore, 1989; Mora et al., 1989; Matteoli et al., 1990), and has been used as a marker for spinal motoneurons in vitro (Juurink et al., 1990). CGRP has also been shown to coexist with acetylcholine in the rat hypoglossal nucleus (Takami et al., 1985).

Functionally, CGRP has been shown to regulate acetylcholine receptor (AChR) synthesis at the neuromuscular junction (New & Mudge, 1986; Fontaine et al., 1986), to stimulate cAMP in developing chick myotubes in culture (Laufer & Changeux, 1987) and to induce normal ultrastructure in dysgenic myotubes (Garcia et al., 1990). These findings support a trophic role for the peptide.

Studies of the response of CGRP immunoreactivity (IR) to motoneuron injury have been limited. However, CGRP-IR has been shown to increase after transection of the facial and sciatic nerves (Streit et al., 1989; Arvidsson et al., 1990). It has been suggested that CGRP is differentially regulated in motor and sensory systems; CGRP-IR increases in facial motoneurons, yet decreases in dorsal root ganglion (DRG) cells after nerve injury (Dumoulin et al., 1990). This evidence, coupled with the putative trophic role of CGRP as described above, warrants further investigation of CGRP's role during motor nerve regeneration.

## RATIONALE

ChAT, the neurotransmitter-synthesizing enzyme for acetylcholine, is present during the normal motoneuron's communicative life. CGRP, a putative trophic factor, appears in late prenatal development; its intensity is greatest at the end of the first postnatal week and reaches adult levels of reduced immunoreactivity by the end of the fourth postnatal week (Kubota et al., 1988). Although morphological and biochemical data have been reported on the response of each of these endogenous substances to peripheral nerve injury, no systematic study has attempted to examine simultaneously how ChAT and CGRP expression is altered after nerve injuries of increasing severity.

The purpose of this work was therefore to examine the role of ChAT, CGRP and their interaction in regeneration and degeneration of hypoglossal motoneurons after nerve crush, transection, or resection. It was hypothesized that severity of injury affects the response of these endogenous substances in the hypoglossal motoneuron. This hypothesis was tested by examining qualitative and quantitative changes in immunoreactivity of ChAT and CGRP at intervals from 1-50 days after nerve injury.

## MATERIALS AND METHODS

### Animal Population

Adult male Sprague-Dawley rats (Charles River) were used (n=26). Rats were housed in the Laboratory Animal Medicine Facility with a day-night cycle of twelve hours, and fed a standard diet of rat pellets and water ad libitum.

### Animal Surgery

Consistent surgical techniques were performed for immunocytochemistry and HRP labeling techniques. Under anesthesia (7% chloral hydrate, 0.5 mg/100 g, i.p.), a midsagittal incision was made at the level of the hyoid bone, the salivary glands were reflected, and the right hypoglossal nerve was exposed from beneath the posterior belly of the digastric muscle (Fig. 1). Three types of nerve injury were performed on the right hypoglossal nerve as it crosses the occipital artery: nerve crush for three minutes with a self-locking needle holder, nerve transection with the cut ends left in apposition, and nerve resection of at least an 8 mm segment of nerve from where it crosses the occipital artery to its bifurcation into medial and lateral branches under the tendon of the digastric muscle (Fig. 2). Following recovery, the rats were allowed to survive for periods of 1, 3, 7, 20 or 50 dpo.

Two rats served as control animals. All procedures were identical to those followed in the experimental series,

**Figure 1. Surgical field. The hypoglossal nerve is isolated with sutures. 8X.**

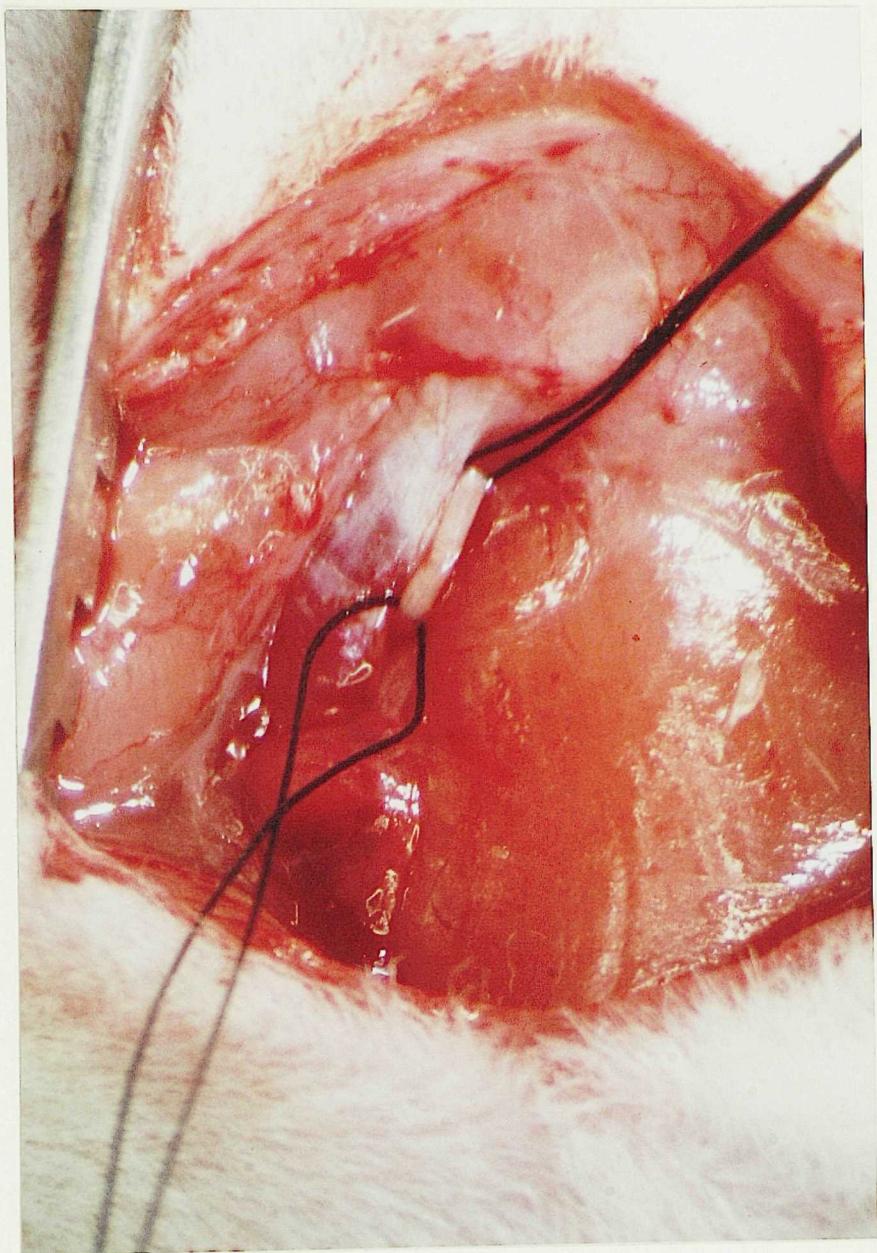
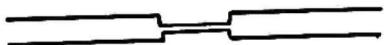
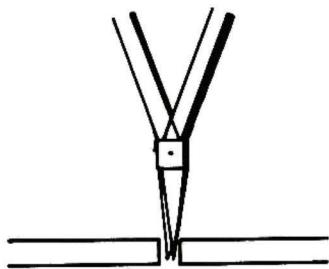


Figure 1

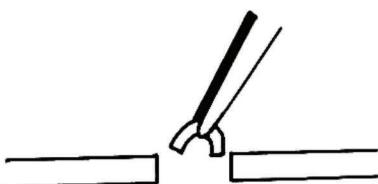
**Figure 2. Types of nerve injury.**



## NERVE CRUSH



## NERVE TRANSECTION



## NERVE RESECTION

Figure 2

except the hypoglossal nerve was not injured.

#### Immunocytochemistry

At the appropriate postoperative times, animals were reanesthetized and killed by intracardiac perfusion, with a prewash of 0.1M phosphate buffer, a fixative of 4% paraformaldehyde/0.1% gluteraldehyde/0.1M phosphate buffer, and a postwash of 10% sucrose/0.1M phosphate buffer. Brains were removed from the cranium, the medulla isolated and the meninges detached before overnight infiltration in a 30% sucrose/0.1M phosphate buffer solution. Tissue blocks were freeze-thawed in isopentane/liquid nitrogen and sectioned through the rostrocaudal extent of the hypoglossal nucleus on a Vibratome at 40-50- $\mu$ m.

#### ChAT Immunocytochemistry

Free floating sections were pretreated with 10% normal goat serum (NGS; Cappel) in Tris buffered saline (TBS) for one hour. Sections were incubated overnight at 4° C in a monoclonal antibody from rat-mouse hybridoma cells directed against choline acetyltransferase (1:8, Boehringer), diluted in 20% NGS/2% bovine serum albumin (BSA) in TBS. Sections were washed in TBS and incubated in biotinylated goat anti-rat IgG (1:50, Boehringer) diluted in 20% NGS/1% BSA/TBS for one hour at room temperature, followed by TBS washes and incubation in Streptavidin-HRP (1:1000, Boehringer) in 0.02% BSA/TBS for one hour at room

temperature. Immunoreactivity was visualized by 3',3'-diaminobenzidine (DAB, 0.05%) and H<sub>2</sub>O<sub>2</sub> (0.005%) in phosphate buffer.

#### CGRP Immunocytochemistry

Sections adjacent to those reacted for ChAT-IR were incubated overnight at room temperature in a polyclonal rabbit CGRP antiserum (1:3000, Cambridge) in 20% NGS/TBS. Sections were washed in TBS, incubated with biotinylated goat anti-rabbit IgG (1:200, Vector Elite ABC kit) in 3% NGS/TBS for two hours at room temperature, washed again and incubated for two hours at room temperature in ABC complex (Elite ABC kit, Vector). DAB was used as the chromagen as described above.

Negative controls for both immunochemical procedures included omitting the primary and secondary antibodies, and the avidin reagent. Positive control experiments consisted of replacing the primary antibody with rat IgG.

#### Immunocytochemistry Quantitation

Quantitation of immunochemically stained tissue sections was performed with a computerized densitometry program (MicroComp Image Analysis System, Southern Micro Instruments, Inc., Atlanta, GA) to assess levels of neuronal ChAT-IR and CGRP-IR. Before measurement of cellular immunoreactivity, the background level of illumination was determined by placing the objective over an area of the

slide that contained no tissue. The program was then set to automatically control for differential illumination across the image by subtracting this measurement from each sample. The average intensity of staining, which included staining in cell bodies as well as neuropil, was measured. Five sections per animal through the rostrocaudal extent of the hypoglossal nucleus were analyzed by systematically aligning each section of a nucleus before data collection. Both sides of the hypoglossal nucleus in each section were measured three times with a cursor controlled rectangle, and the average was determined.

A difference in average intensity was determined by subtracting the average intensity of the unoperated side from the average intensity of the operated side. This method was chosen to minimize the inherent variation present between the immunostaining of tissue samples.

50 dpo material was not quantitatively analyzed for ChAT-IR or CGRP-IR. Therefore, the experimental design for assessing changes in the intensity of immunoreactivity was comprised of one group of control (unoperated) rats, and 12 groups of operated rats that received one of three nerve injuries and then were sacrificed on 1, 3, 7 or 20 dpo. Due to the large number of tissue sections generated in this study (130 sections from 13 experimental conditions), the samples of tissue derived from each pair of rats per group were treated as ten independent assessments of nerve injury.

### Statistical Analysis

Tissue samples derived from control animals were analyzed with a paired t-test for a comparison of the average intensity of the two sides of the hypoglossal nucleus (Winer, 1971).

In order to determine any differences in the three types of injuries performed (3 factors) and their effect at different time periods after injury (4 factors), an analysis of variance was performed. The modified factorial design (3-by-4 plus 1 design) outlined in Winer (1971) was used, as this design allows comparisons to the single control group. A priori individual comparisons were performed between the different treatment groups, using the within-cell mean square error term calculated in the analysis of variance as described by Winer (1971).

### HPR Labeling

In order to correlate reinnervation of tongue musculature after axonal damage with changes seen in ChAT and CGRP immunoreactivity, three types of nerve injuries were performed on nine animals as detailed previously. Animals were allowed to survive for 3, 20 and 50 dpo. The rats were reanesthetized and a 10% solution of HRP in 0.1M Tris buffer was injected bilaterally with a Hamilton syringe into the tongue musculature. Three injections per side totalling 75  $\mu$ l were given for two days. Animals were killed by perfusion with a 1 ml injection of heparin into

the left ventricle followed by Ringer's solution, dilute and concentrated aldehydes, and 0.1M phosphate buffer. The medulla was isolated and transverse sections were cut at 50  $\mu$ m on a Vibratome through the rostrocaudal extent of the hypoglossal nucleus on a Vibratome. Sections were processed by the DAB-glucose-oxidase method (Itoh et al., 1979) and counterstained with neutral red. Labeled and unlabeled cells were counted in every third section of each animal for the injured as well as uninjured hypoglossal nuclei. The percent labeling in each nucleus was defined as:

% HRP labeling per hypoglossal nucleus = (# of HRP positive neurons within a hypoglossal nucleus/ total # of neurons counted within the hypoglossal nucleus) X 100.  
The contralateral nucleus served as a control for percent normal labeling.

## RESULTS

### CHOLINE ACETYLTRANSFERASE IMMUNOREACTIVITY

#### Qualitative Observations of Hypoglossal Nuclei

##### Unoperated Animals

Intense ChAT immunoreactivity was detected in hypoglossal nuclei of each side in uninjured rats (Fig. 3). ChAT-IR was distributed in neuronal somata, the surrounding neuropil and XII nerve fibers exiting the ventrolateral aspect of the nucleus.

##### Hypoglossal Nuclei Contralateral to Axotomy

ChAT-IR was observed in neuronal somata and neuropil of hypoglossal nuclei as well as within the exiting hypoglossal nerve fibers. ChAT-IR appeared comparable in intensity and distribution to the hypoglossal nuclei of unoperated control animals (See Fig. 4a).

##### Hypoglossal Nuclei Ipsilateral to Axotomy

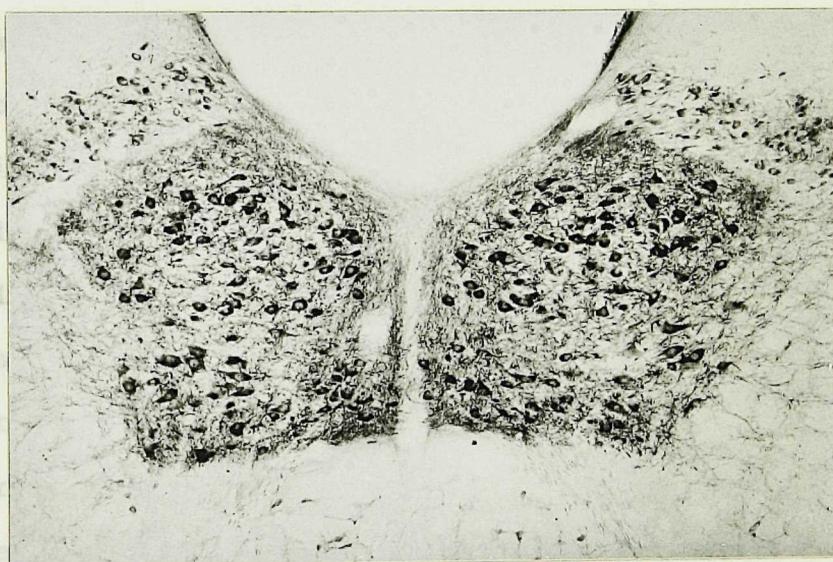
A decrease in ChAT-IR in somata of hypoglossal neurons and neuropil occurred initially at 3 dpo. At this time the extent of the loss of ChAT-IR was related to the nature of the injury: a lesser reduction in ChAT-IR was detected after nerve crush than after transection and resection, whereas in the latter cases the loss of ChAT-IR seemed comparable. At 7 dpo, the maximal loss of ChAT-IR

Figure 3. Normal ChAT-IR, control (unoperated) rat. Both sides of hypoglossal nucleus have equal IR.

All photographs show true left and right; 82X.



was observed, and the extent of the depletion continued to relate to the nature of nerve injury. Fewer ChAT reactive somata and less immunoreactivity in the neuropil characterized the hypoglossal nuclei of nerve crush specimens (Fig. 4a). With transection and resection injuries, a dramatic depletion in ChAT-IR was found (Fig. 4b,c). Changes were manifested as an almost complete loss of IR in the neuropil, and neuronal cell bodies appeared ghost-like (Fig. 5a,b). By 20 dpo, ChAT-IR had returned to that demonstrated in the contralateral nucleus for nerve



crush cases (Fig. 6a). At 20 dpo, ChAT-IR had returned to that demonstrated in the contralateral nucleus for nerve crush cases (Fig. 6b). By 40 dpo, the number and size of somata had returned to that demonstrated in the contralateral nucleus for nerve crush cases (Fig. 6c). At 60 dpo, the number and size of somata and neuropil staining had returned to that demonstrated in the contralateral nucleus for nerve crush cases (Fig. 7a). At 120 dpo, the number and size of somata and the reduction in the cross sectional area of the nucleus on the nerve injured side (Fig. 7b,c).

#### Quantitative Immunocytochemistry

#### Average Intensity in Uninjured versus Uninjured Hypoglossal Nuclei

A paired t-test indicated no significant difference in ChAT-IR between sides. **Figure 3** hypoglossal nucleus (see

was observed, and the extent of the depletion continued to relate to the nature of nerve injury. Fewer ChAT reactive somata and less immunoreactivity in the neuropil characterized the hypoglossal nuclei of nerve crush specimens (Fig. 4a). With transection and resection injuries, a dramatic depletion in ChAT-IR was found (Fig. 4b,c). Changes were manifested as an almost complete loss of IR in the neuropil, and neuronal cell bodies appeared ghost-like (Fig. 5a,b). By 20 dpo, ChAT-IR had returned to that demonstrated in the contralateral nucleus for nerve crush cases (Fig. 6a). With nerve transection, a meager return of IR was seen in few somata and staining was still absent in the neuropil (Fig. 6b). ChAT-IR remained virtually unchanged for nerve resection at 20 dpo; only a few cell bodies had regained ChAT-IR, and the cross-sectional area of injured nuclei had decreased in size (Fig. 6c). At 50 dpo, normal levels of ChAT-IR in neuronal somata and neuropil were demonstrated for all three types of injury (Fig. 7a). However, the resected nuclei displayed a reduction in the cross sectional area of the nucleus on the nerve injured side (Fig. 7b,c).

#### Quantitative Immunocytochemistry

##### Average Intensity in Injured versus Uninjured Hypoglossal Nuclei

A paired t-test indicated no significant difference in ChAT-IR between sides of the hypoglossal nucleus (see

Figure 4. ChAT-IR, 7 dpo. Maximal neuronal response in the hypoglossal nucleus to nerve injury. A. nerve crush B. nerve transection C. nerve resection

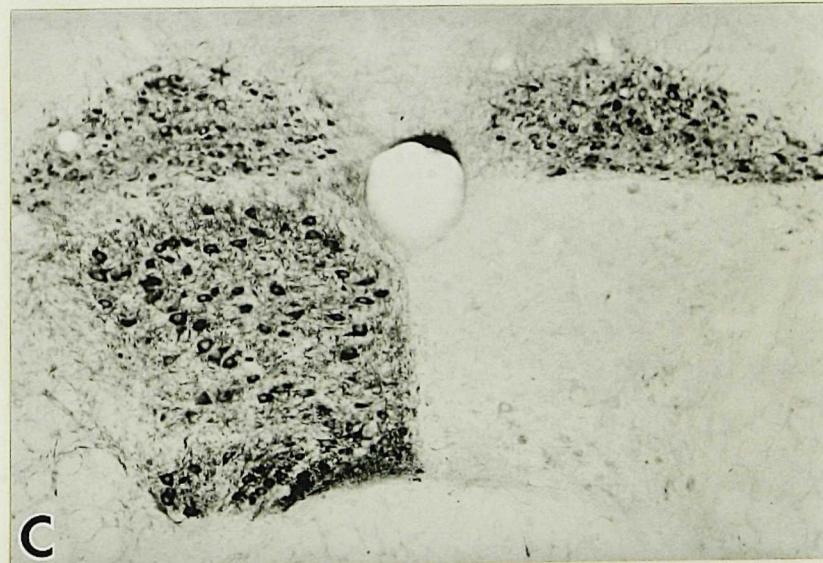
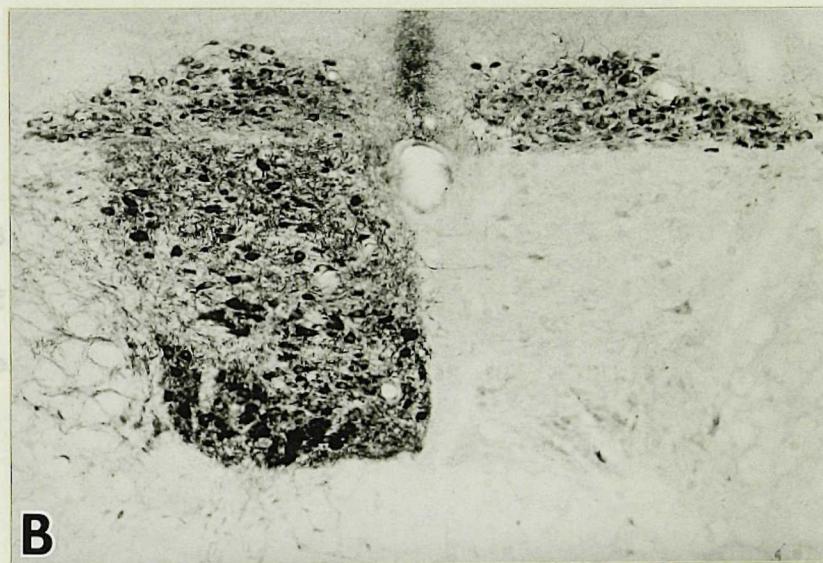
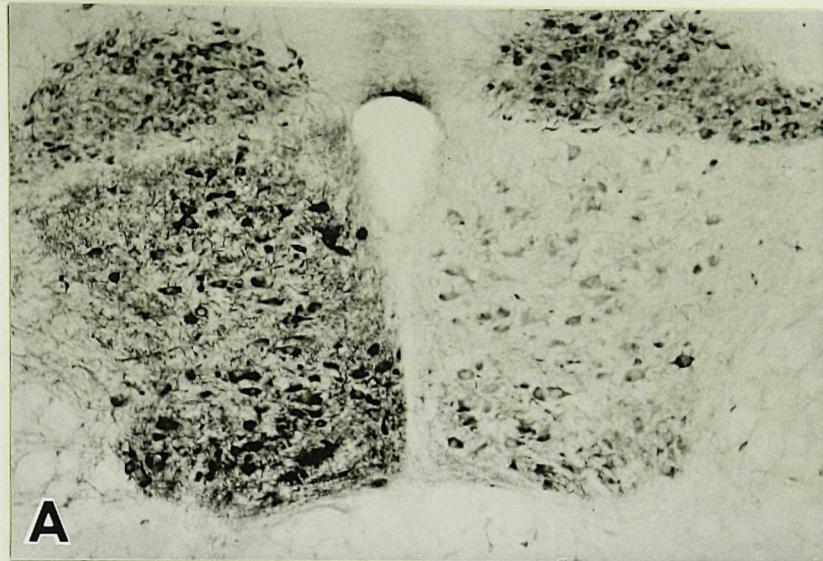


Figure 4

Figure 5  
sides of  
A. unope  
330X

**Figure 5. ChAT-IR, 7 dpo. Comparison of left and right sides of hypoglossal nucleus after nerve transection.**

**A. unoperated side (L) B. operated side (R)**

**330X.**



Figure 5

hypoglossal nucisi operculi nervi

A. nerve crush B. nerve transected  
resection

Figure 5

Figure 6. ChAT-IR, 20 dpo. Return of ChAT-IR in operated hypoglossal nuclei apparent for nerve crush and transection.

A. nerve crush   B. nerve transection                    C. nerve  
resection

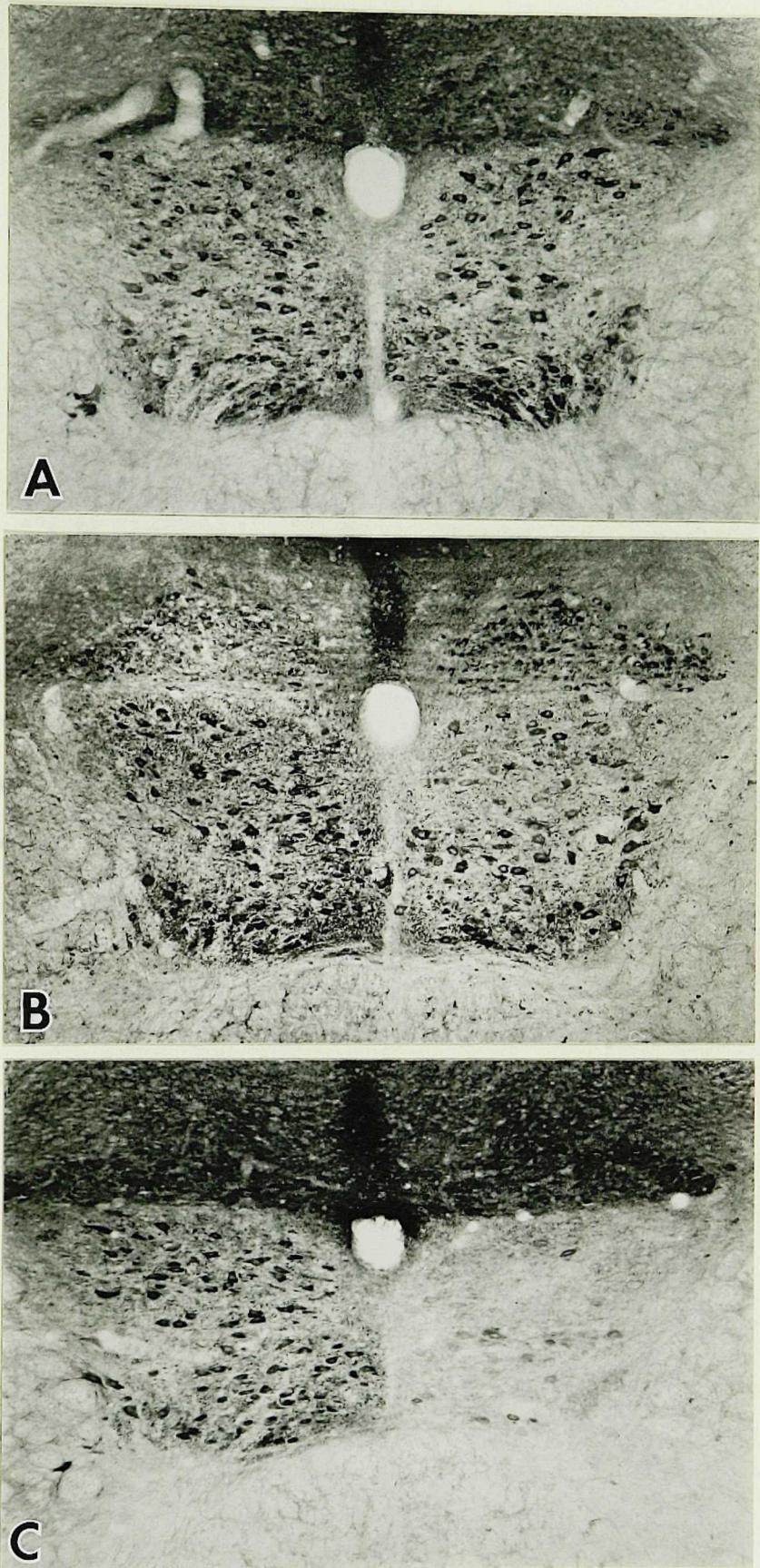


Figure 6

Figure 7. ChAT-IR, 50 dpo. Return of normal ChAT-IR for all three injuries. A. nerve crush B. nerve transection C. nerve resection

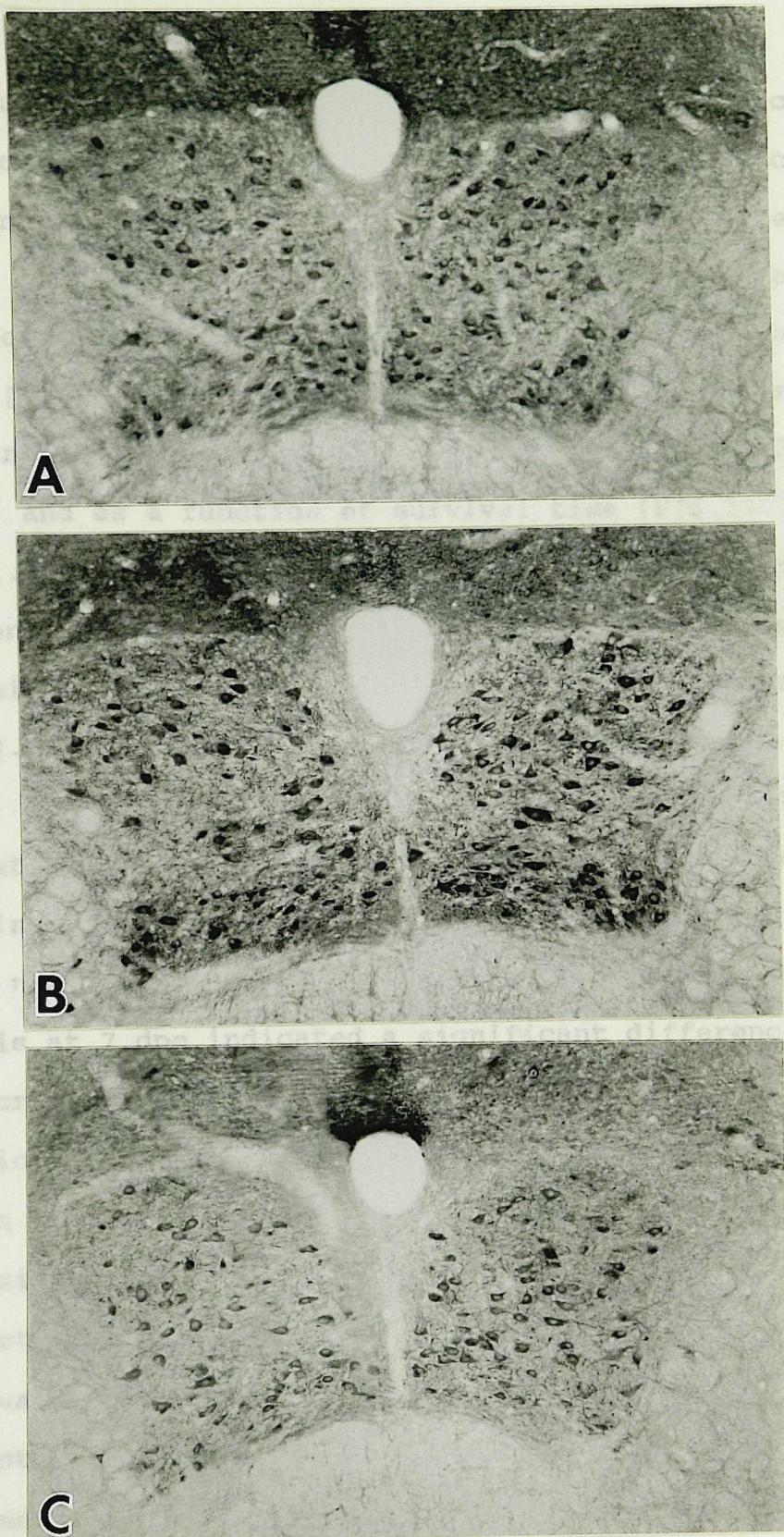


Figure 7. Effect of nerve crush on nerve regeneration.

Fig. 3) in the control group [ $t(9)=0.92$ ;  $p=0.382$ ].

Therefore, subsequent analyses utilized different scores to assess changes in neuronal intensity as a consequence of nerve injury compared with staining in the unoperated side.

Analysis of variance indicated that there was a significant difference between control animals and all other groups [ $F(1,117)=28.0$ ;  $p<0.001$ ], as well as differences resulting from different injury types [ $F(2,117)=7.32$ ;  $p<0.01$ ] and as a function of survival time [ $F(3,117)=2.78$ ;  $p<0.05$ ]. There were also changes in staining intensity for different injury types that were dependent upon length of survival time [the interaction effect:  $F(6,117)=2.78$ ;  $p<0.05$ ].

Individual comparisons were performed to further delineate changes in staining intensity at each time point after injury (See Fig. 8). Analysis of 1 and 3 dpo data showed no significant differences between injury types. Analysis at 7 dpo indicated a significant difference between nerve crush and transection and between nerve crush and resection. However, there was no significant difference between nerve transection and resection. At 20 dpo, there was a significant difference between nerve crush and transection and between nerve crush and resection. Again, there was no significant difference between nerve transection and resection. A summary of derived F-values for these comparisons is presented in Table I (CR-nerve crush; TR-nerve transection; RE-nerve resection).



Figure 8. Difference in average intensity between uninjured (L) and injured (R) sides of hypoglossal nuclei for three types of nerve injury as a function of postoperative time (Mean +/- Standard Error of the Mean).

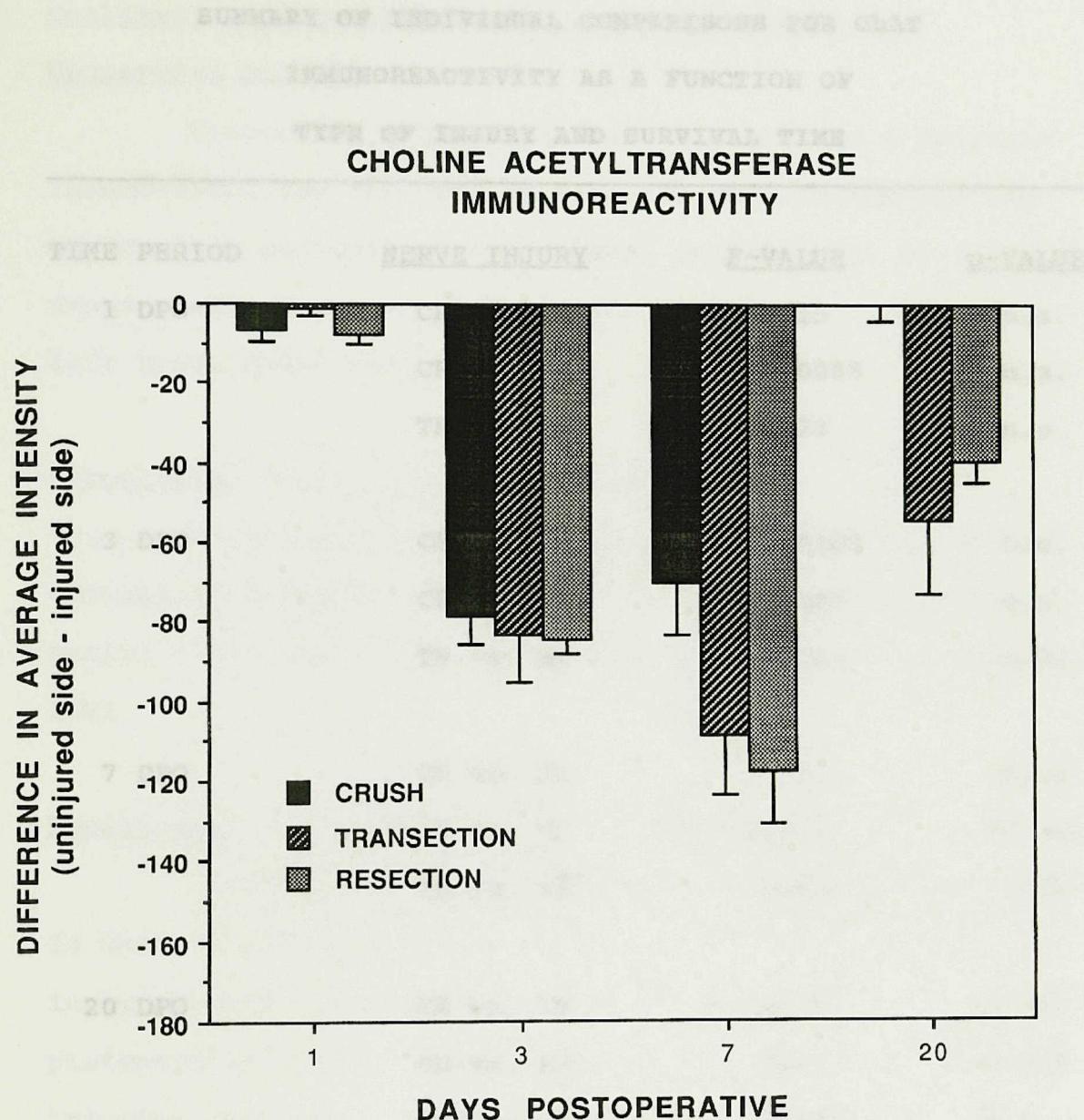


Figure 8

Table I

**SUMMARY OF INDIVIDUAL COMPARISONS FOR ChAT  
IMMUNOREACTIVITY AS A FUNCTION OF  
TYPE OF INJURY AND SURVIVAL TIME**

<u>TIME PERIOD</u>	<u>NERVE INJURY</u>	<u>F-VALUE</u>	<u>p-VALUE</u>
1 DPO	CR vs. TR	0.15	n.s.
	CR vs. RE	0.0085	n.s.
	TR vs. RE	0.23	n.s.
3 DPO	CR vs. TR	0.0122	n.s.
	CR vs. RE	0.186	n.s.
	TR vs. RE	0.294	n.s.
7 DPO	CR vs. TR	7.82	<0.01
	CR vs. RE	11.97	<0.001
	TR vs. RE	0.44	n.s.
20 DPO	CR vs. TR	16.14	<0.001
	CR vs. RE	8.50	<0.005
	TR vs. RE	1.215	n.s.

n.s.: not significant

## CALCITONIN GENE-RELATED PEPTIDE IMMUNOREACTIVITY

### Qualitative Observations of Hypoglossal Nuclei

#### Unoperated Animals

Unoperated control animals demonstrated a moderate immunoreactivity for CGRP in neuronal cell bodies and the surrounding neuropil of hypoglossal nuclei (Fig. 9). Immunostaining was of similar intensity in the right and left hypoglossal nuclei.

#### Hypoglossal Nuclei Contralateral To Axotomy

At all postoperative time periods there was no obvious differences in intensity of CGRP-IR between the nuclei contralateral to injury and control animals (See Fig. 10a).

#### Hypoglossal Nuclei Ipsilateral to Axotomy

A change in CGRP-IR was apparent before any changes in ChAT-IR were detectable. CGRP-IR increased dramatically in hypoglossal nuclei ipsilateral to axotomy at postoperative days 1-3 and appeared the same for all injuries (10a,b,c). Intense staining of fibers in the neuropil and increased content in neuronal cell bodies characterized this change (Fig. 11a,b). This significant increase in CGRP-IR was maintained at 7 dpo in nerve crush specimens. In contrast to these findings, at 7 dpo after nerve transection and resection, CGRP-IR appeared equal in

Figure 9. Normal CGRP-IR, control (unoperated) rat. Both sides of hypoglossal nucleus have equal IR.

Figure 9

Ductal

C. nect.

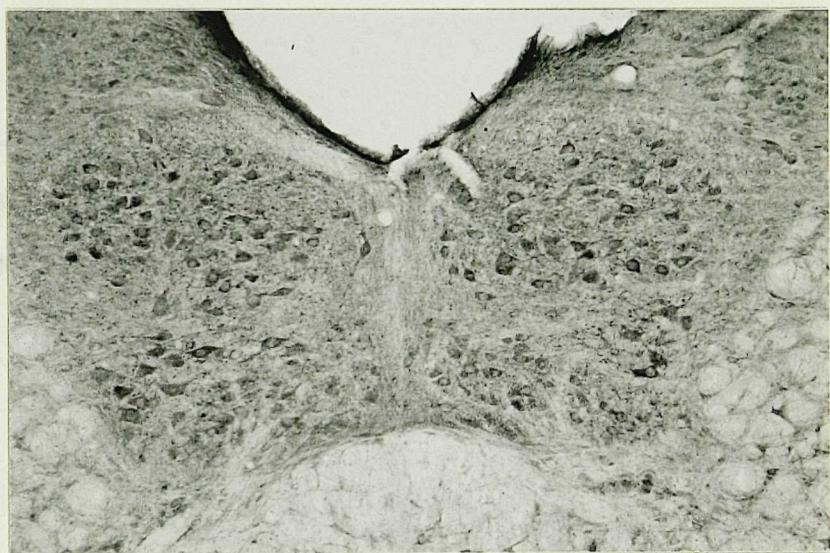


Figure 9

Figure 10. CGRP-IR, 1-3 dpo. Maximal neuronal response to  
nerve injury. A. nerve crush B. nerve transection  
C. nerve resection

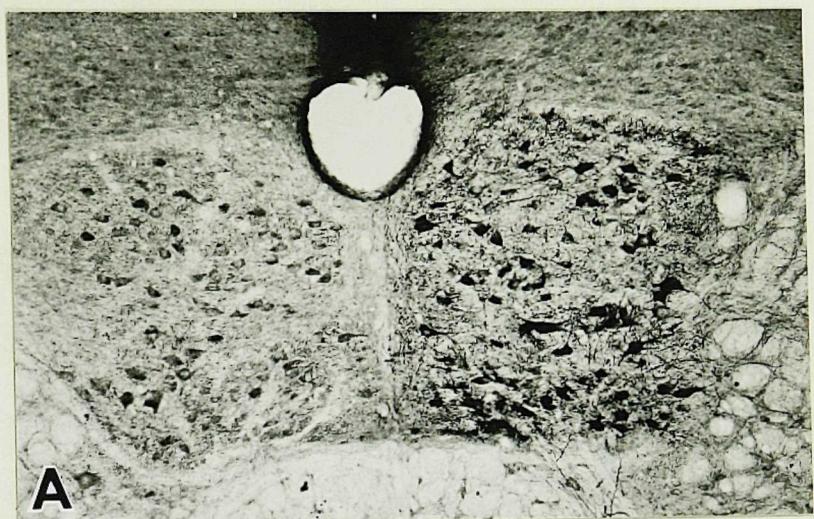
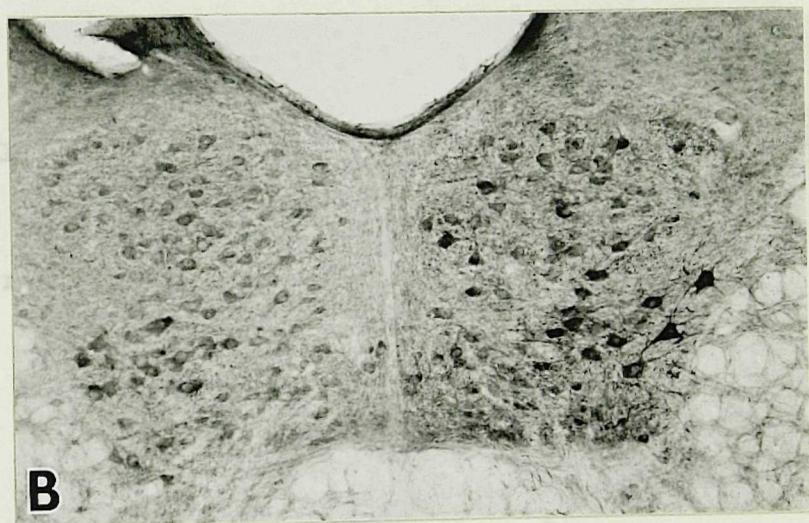
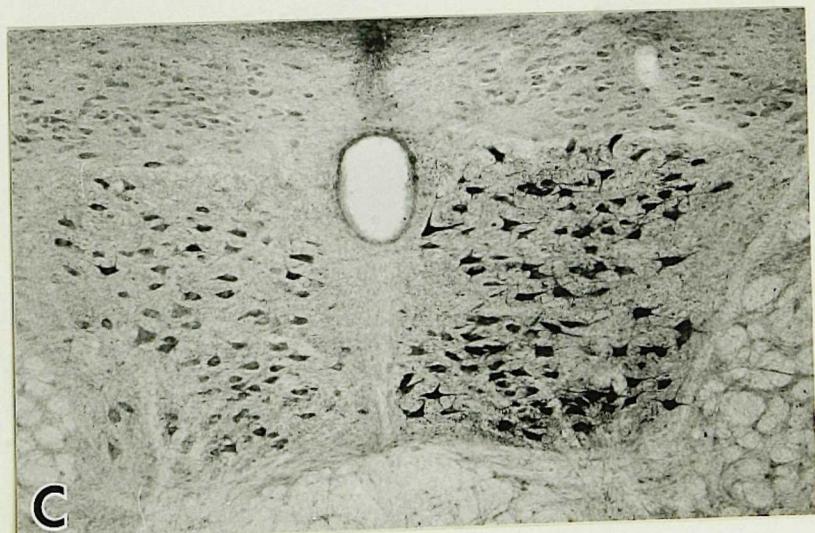
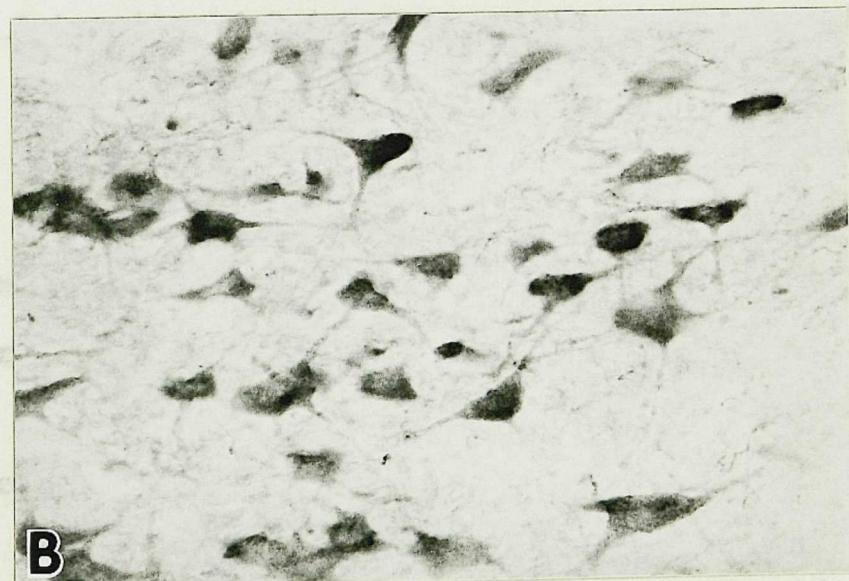
**A****B****C**

Figure 10

Figure 10  
sides of  
A. unope  
330X.

Figure 11. CGRP-IR, 7 dpo. Comparison of left and right sides of hypoglossal nucleus after nerve crush.

A. unoperated side (L) B. operated side (R)  
330X.



**Figure 11** are also shown. In

both sides of the nucleus. At 20 dpo, the CGRP-IR increase in neuronal cell bodies and the surrounding neuropil was maintained in nerve crush specimens (Fig. 12a). At 20 dpo, nerve transection and resection were similar; CGRP-IR remained elevated in cell somata (Fig. 12b,c). The qualitative appearance of transection and resection specimens at 20 dpo seemed that the CGRP-IR was increased on the side contralateral to axotomy. By 50 dpo, nerve crush CGRP-IR was equal on both sides; nerve transection and resection CGRP-IR in the neuropil was at normal levels, with some darkly staining somata (Fig. 13a,b,c). In resection cases, the reduced cross-sectional area of the nucleus was apparent.

#### Quantitative Immunocytochemistry

##### Average Intensity of Injured versus Uninjured Hypoglossal Nuclei

A paired t-test showed no significant difference in CGRP-IR between sides of the hypoglossal nucleus (see Fig. 9) in the unoperated control group [ $t=0.269$ ;  $p=0.794$ ].

Therefore

Analysis of variance indicated there was a significant difference in staining intensity between control animals and all other groups [ $F(1,117)=15.72$ ;  $p<0.001$ ], as well as differences resulting from different injury types [ $F(2,117)=11.29$ ;  $p<0.01$ ] and as a function of survival time [ $F(3,117)=27.87$ ;  $p<0.001$ ]. There were also changes in

Figure 12. CGRP-IR, 20 dpo. Sustained elevation of CGRP-IR  
in A; B and C show decreased CGRP-IR.                    A. nerve crush  
B. nerve transection    C. nerve resection

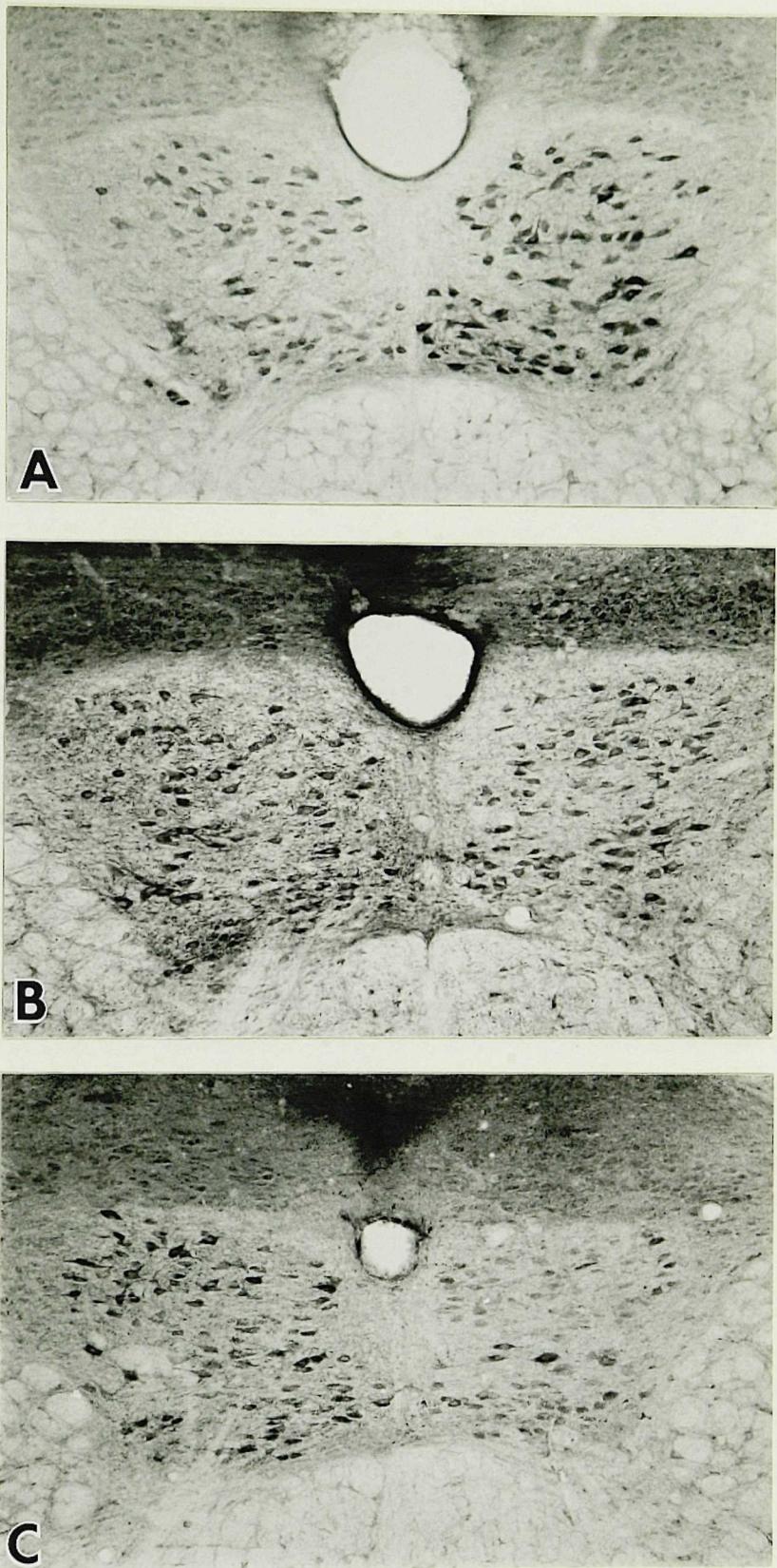


Figure 12

Figure 13. CGRP-IR, 50 dpo. Equal IR in left and right sides in A; decreased IR in right side of hypoglossal nucleus with some immunoreactive somata in B, C.  
A. nerve crush B. nerve transection C. nerve resection

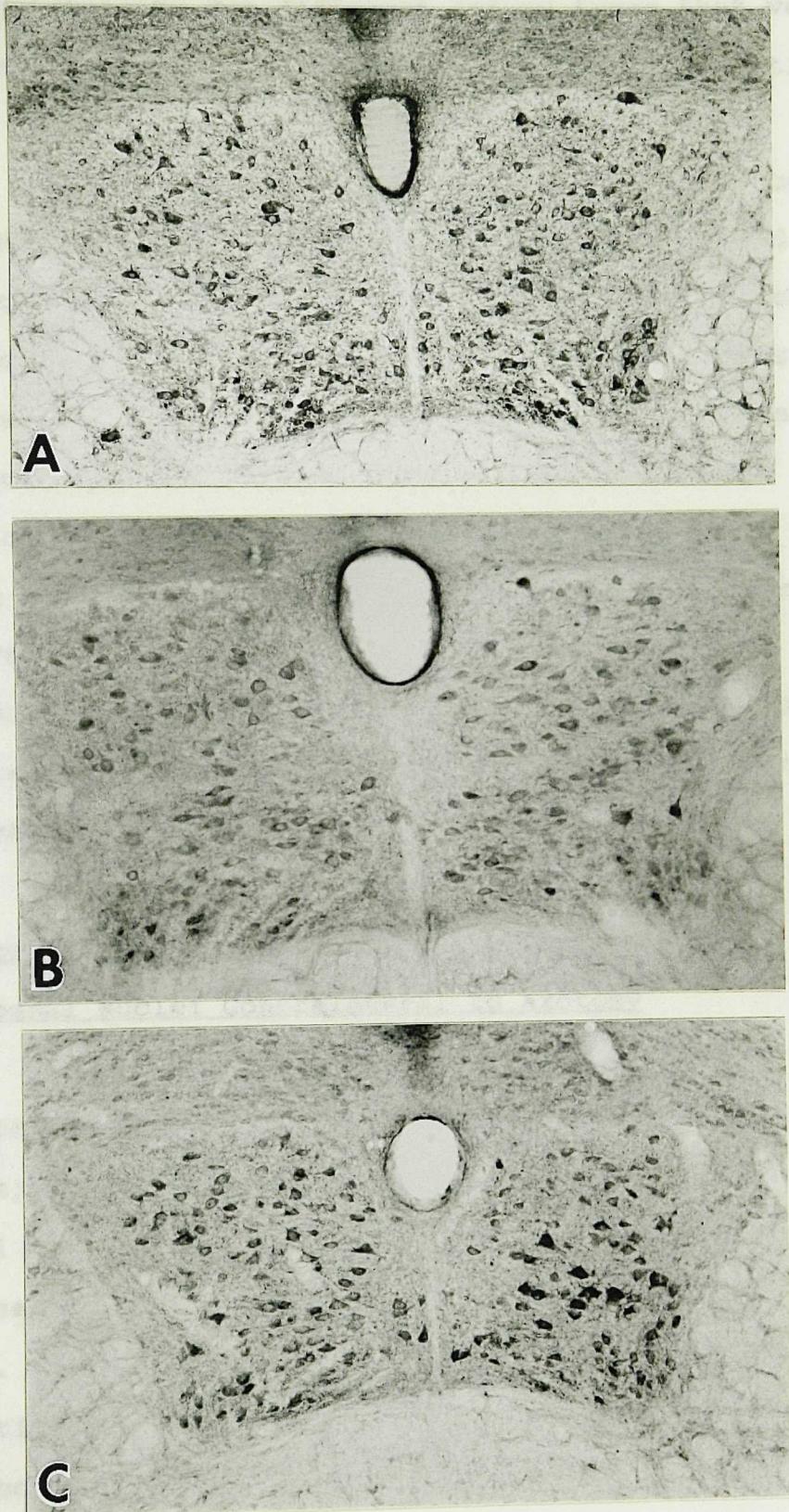


Figure 13

staining intensity for different injury types that were dependent upon length of survival time [the interaction effect:  $F(6,117)=21.61$ ;  $p<0.001$ ].

Individual comparisons indicated no significant differences between tissue samples obtained at 1 and 3 dpo (See Fig. 14). At 7 dpo, statistical analysis showed a significant difference between nerve crush and transection and between nerve crush and resection. No significant difference was found between nerve transection and resection. At 20 dpo, a significant difference was found between all three nerve injury types: crush vs. transection, crush vs. resection, and transection vs. resection. A summary of derived F-values for these comparisons is presented in Table II (CR-nerve crush; TR-nerve transection; RE-nerve resection).

#### HRP Labeling of Hypoglossal Nuclei

##### Hypoglossal Nuclei Contralateral to Axotomy

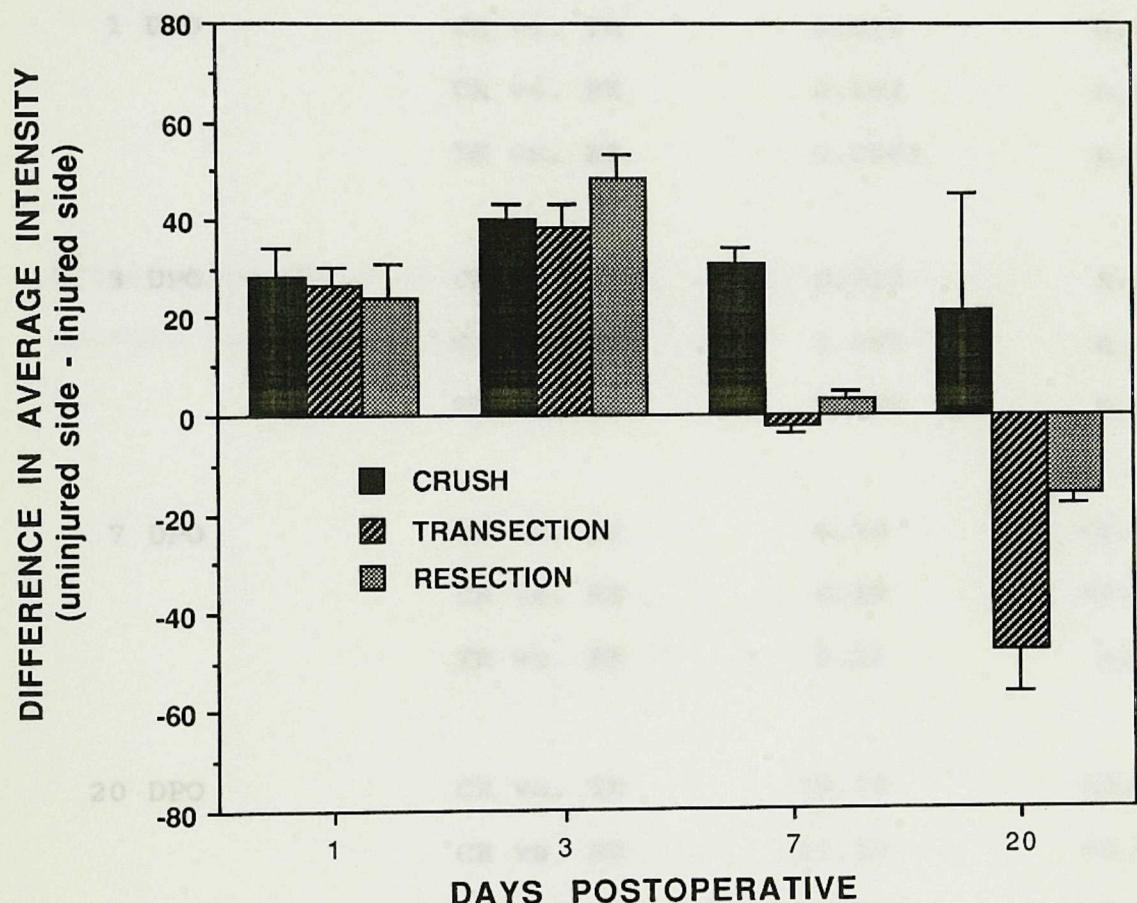
Hypoglossal nuclei contralateral to axotomy demonstrated HRP labeling of neurons at the three survival periods: 3, 20, and 50 days. An average of 67% HRP labeling in cell bodies was found in these nuclei (Fig. 15), which is in agreement with Hall (1988) for uninjured hypoglossal nuclei. It has been documented that there is an interneuron population in the hypoglossal nucleus that does not take up HRP (Boone & Aldes, 1984). Therefore, this value served as

Figure 14. Difference in average intensity between uninjured (L) and injured (R) sides of hypoglossal nuclei for three types of nerve injury as a function of postoperative time (Mean +/- SEM).

Table 11

SUMMARY OF INDIVIDUAL COMPARISONS AND TESTS  
IMMUNOREACTIVITY AT 1, 3, 7, AND 20 DPO

**CALCITONIN GENE-RELATED PEPTIDE  
IMMUNOREACTIVITY**



n.s.: not significant

Figure 14

Table II

**SUMMARY OF INDIVIDUAL COMPARISONS FOR CGRP**  
**IMMUNOREACTIVITY AS A FUNCTION OF**  
**TYPE OF INJURY AND SURVIVAL TIME**

<u>TIME PERIOD</u>	<u>NERVE INJURY</u>	<u>F-VALUE</u>	<u>p-VALUE</u>
1 DPO	CR vs. TR	0.019	n.s.
	CR vs. RE	0.142	n.s.
	TR vs. RE	0.0568	n.s.
3 DPO	CR vs. TR	0.023	n.s.
	CR vs. RE	0.602	n.s.
	TR vs. RE	0.863	n.s.
7 DPO	CR vs. TR	8.70	<0.005
	CR vs. RE	6.19	<0.05
	TR vs. RE	0.21	n.s.
20 DPO	CR vs. TR	38.78	<0.001
	CR vs. RE	11.15	<0.005
	TR vs. RE	8.34	<0.01

n.s.: not significant

Figure 15. Ratio of HRP-labeled neurons/total number of neurons counted among injured animals.

*HRP Labeling of Hypoglossal Nuclei*

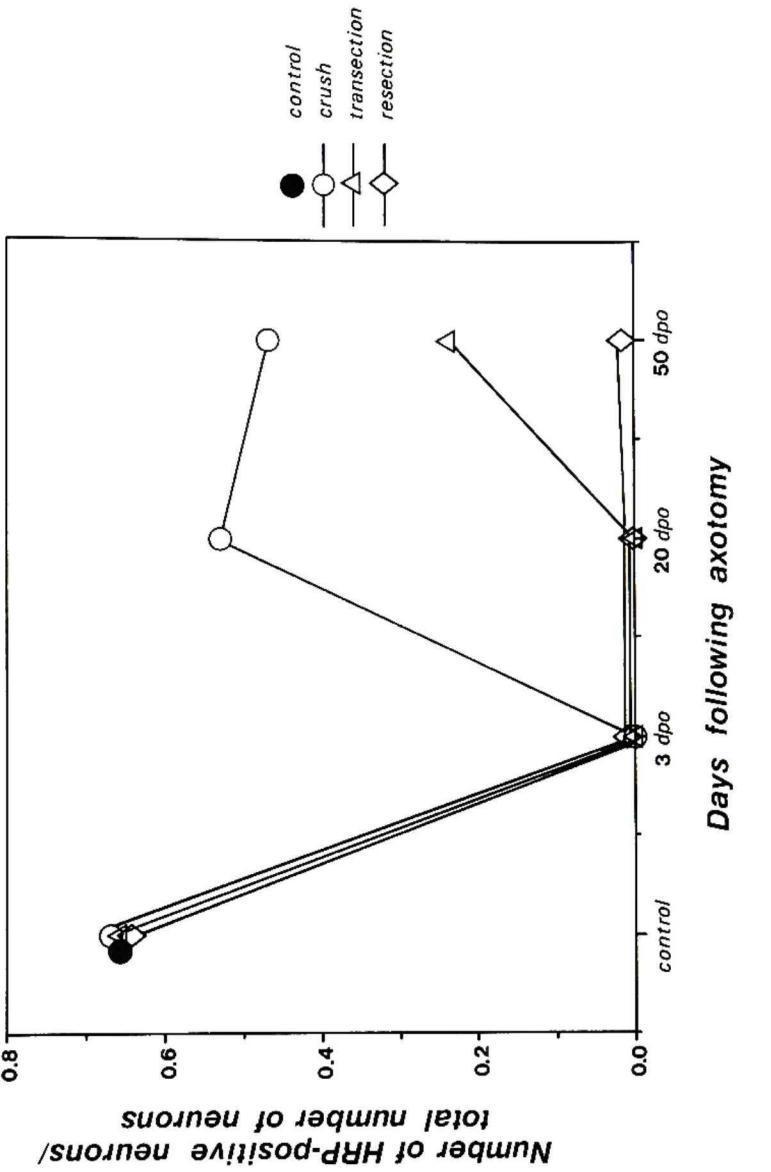


Figure 15

a control.

Hypoglossal Nuclei Ipsilateral to Axotomy

At 3 dpo, the earliest time period examined, no HRP labeling of neurons in the ipsilateral nucleus was obtained after injections of the exogenous protein into the tongue musculature. At 20 dpo after nerve crush injury (Fig. 16) 53% of the neurons in the ipsilateral nucleus contained HRP, whereas no HRP labeled neurons were detected for nerve transection (Fig. 17) and resection cases (Fig. 18). The level of HRP labeled neurons was maintained at 50 dpo for the nerve crush (Fig. 19). At 50 dpo, 24% of the neurons in the ipsilateral hypoglossal nucleus were retrogradely labeled with HRP in the nerve transected rats (Fig. 20), and only 1% of the neurons whose axons were resected (Fig. 21) contained HRP (See Fig. 15).

Figure 16. Dark field micrograph of retrograde HRP labeling of hypoglossal nuclei 20 days after nerve crush. For all dark field micrographs, injured side is on the right; 125X.

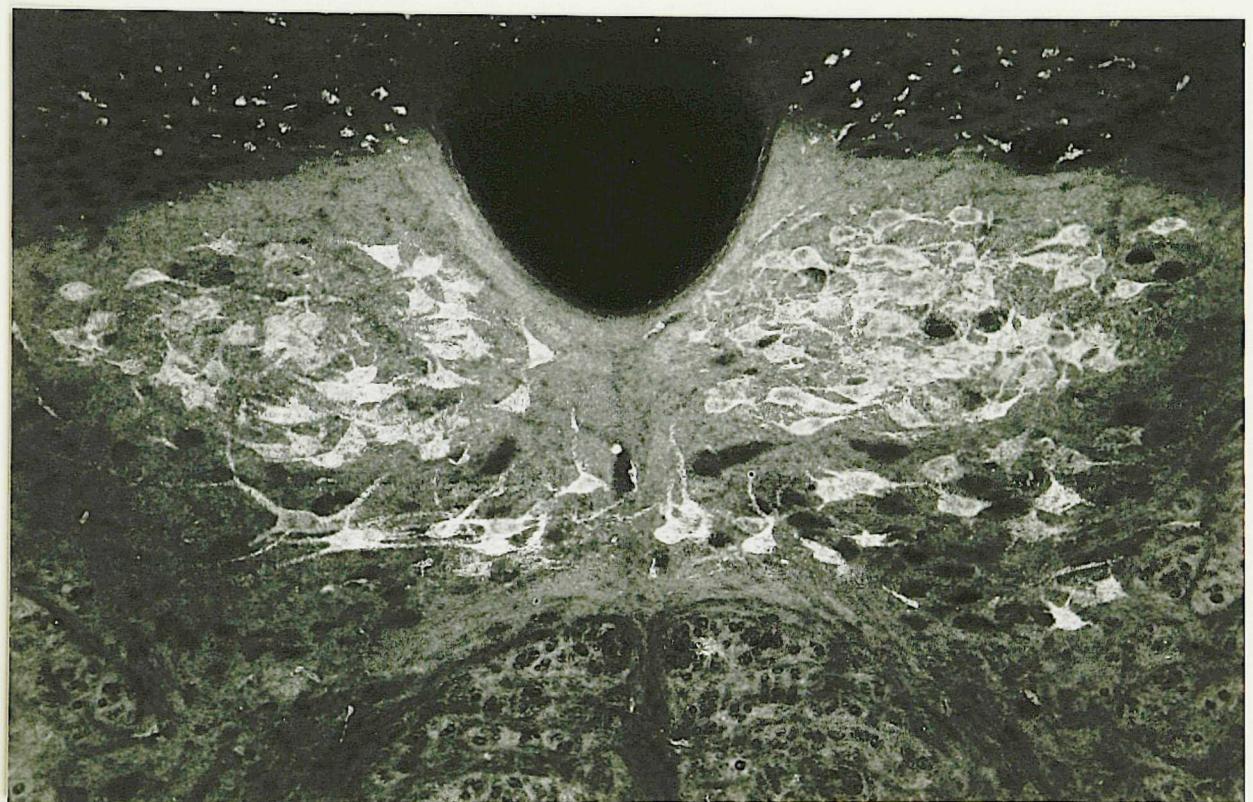


Figure 16

**Figure 17. Dark field micrograph of retrograde HRP labeling of hypoglossal nuclei 20 days after nerve transection.**

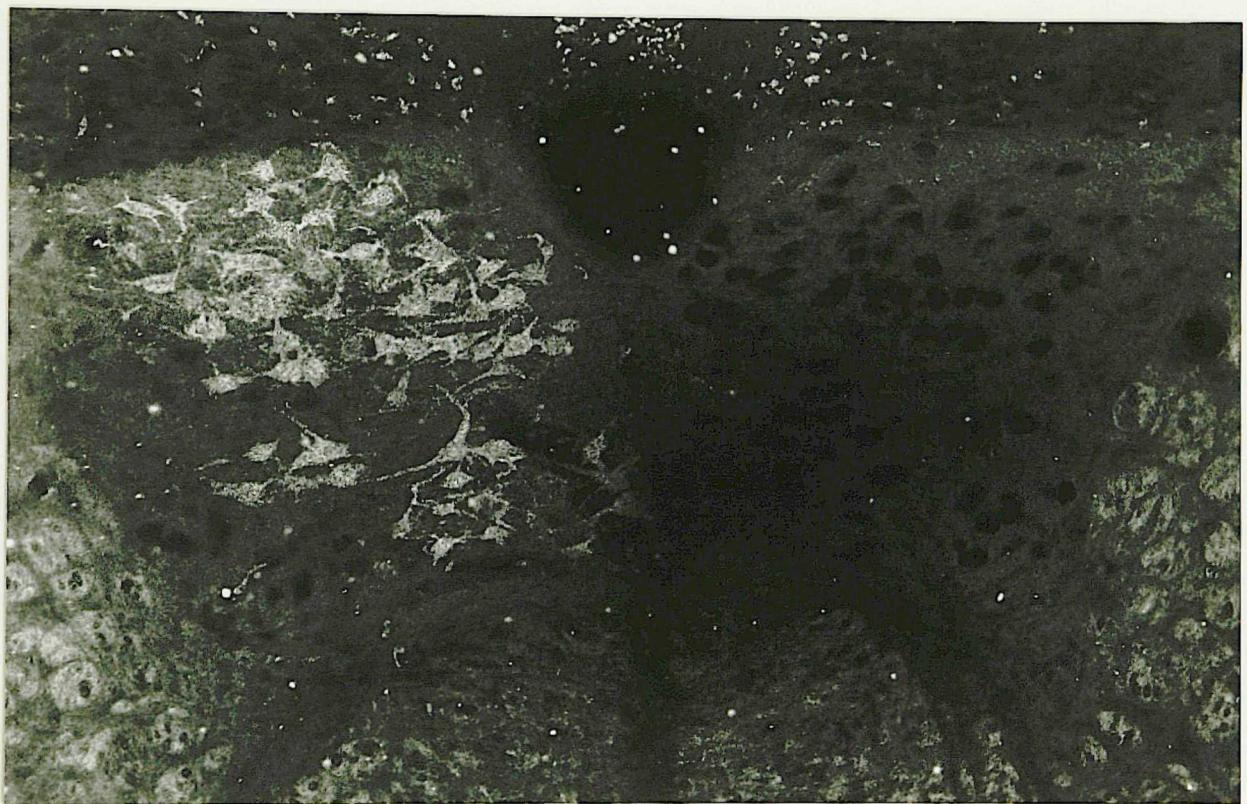
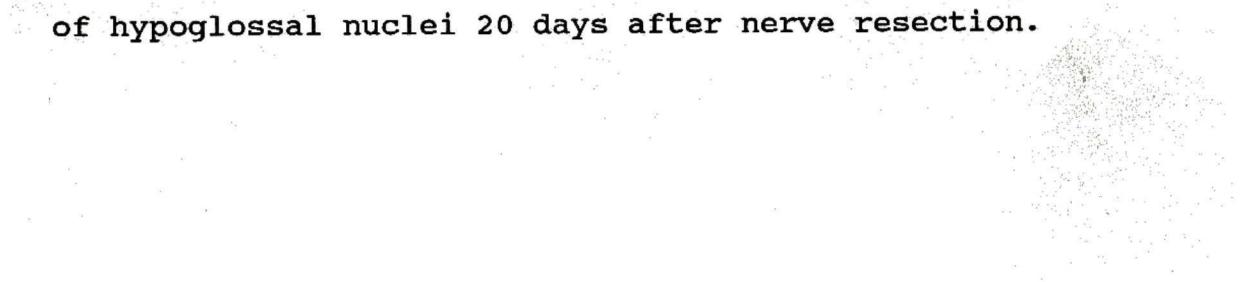


Figure 17

Figure 18. Dark field micrograph of retrograde HRP labeling of hypoglossal nuclei 20 days after nerve resection.



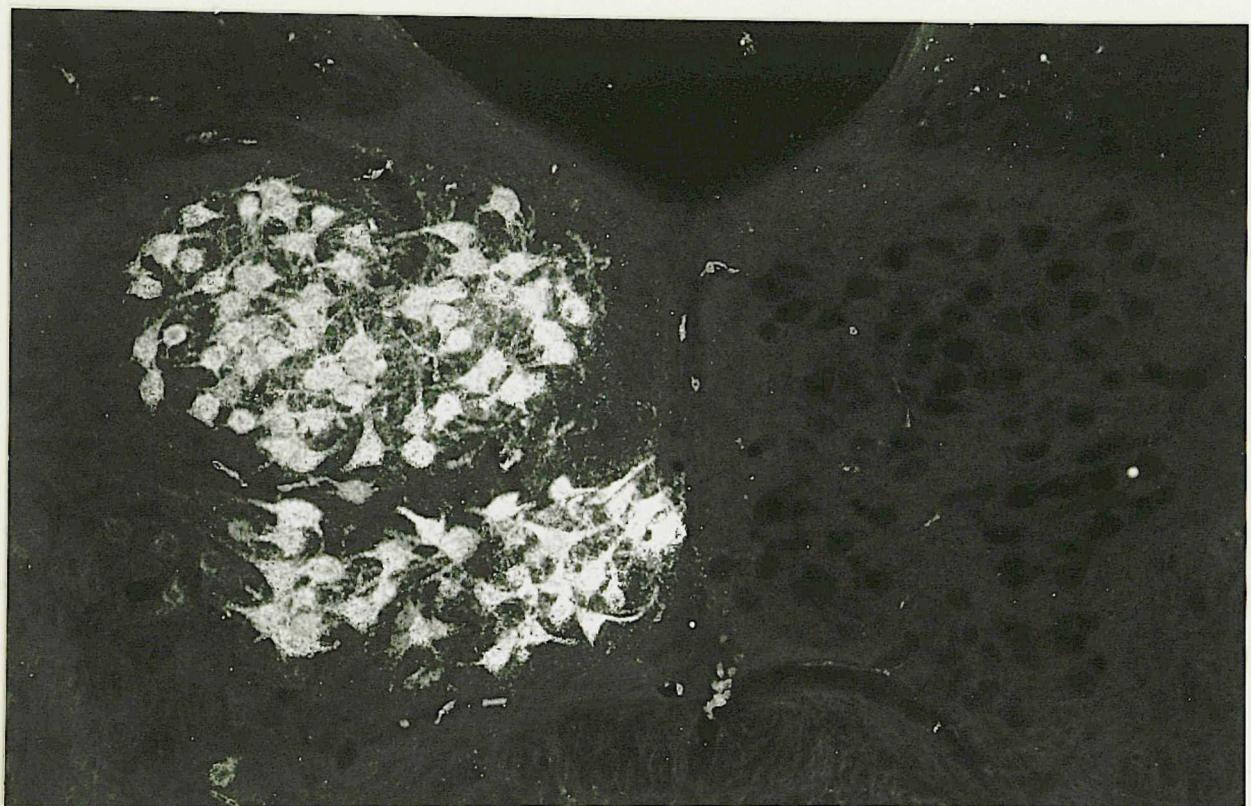
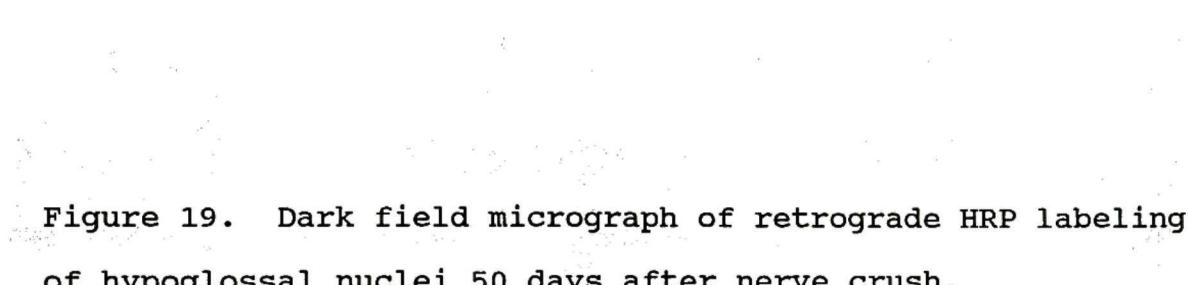


Figure 18



**Figure 19.** Dark field micrograph of retrograde HRP labeling of hypoglossal nuclei 50 days after nerve crush.

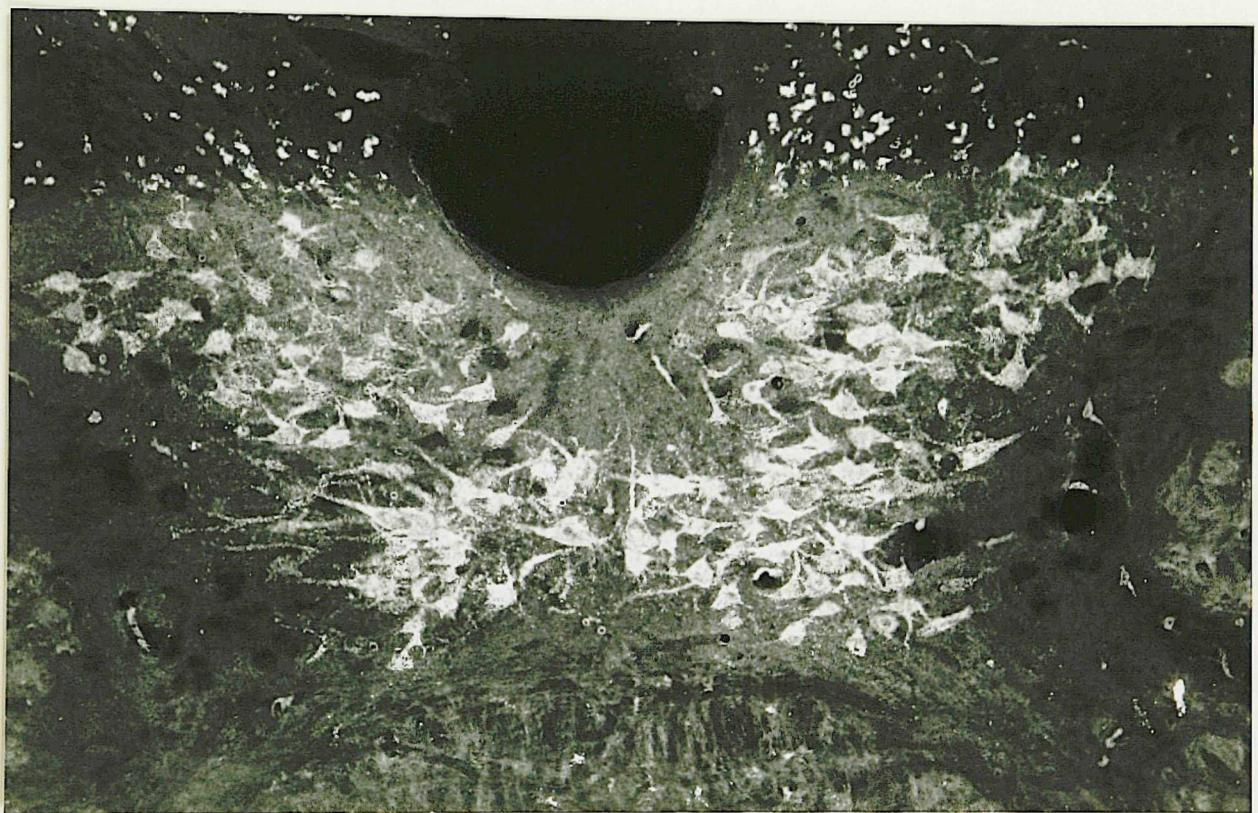


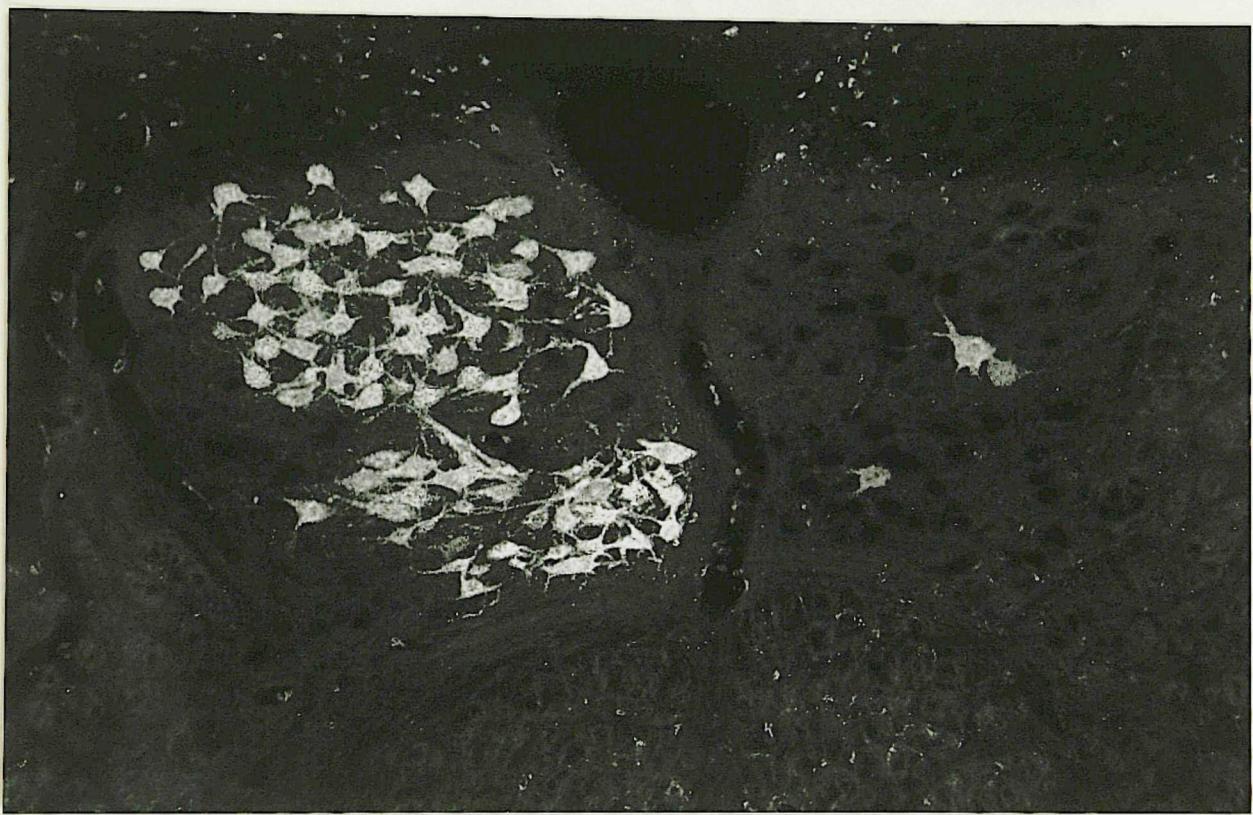
Figure 19

**Figure 20. Dark field micrograph of HRP labeling of hypoglossal nuclei 50 days after nerve transection.**



Figure 20

**Figure 21. Dark field micrograph of HRP labeling of hypoglossal nuclei 50 days after nerve resection.**



changes in the nerve fiber density and the density were found to be correlated with the time of regulation of CGRP and CR. The magnitude of regulation was related to the extent of the injury but not, but not for CGRP. The other difference is the late response to nerve injury instead of the persistence of increased CGRP-IR was followed by regeneration and

**Figure 21**

## DISCUSSION

ChAT, the biosynthetic enzyme of the classical neurotransmitter acetylcholine, and CGRP, a putative trophic neuropeptide, coexist in the neurons of the hypoglossal nucleus (Takami et al., 1985). Previous studies, utilizing a variety of CNS cell groups, have observed how cellular levels of either ChAT (Lams et al., 1988; Armstrong et al., 1991) or CGRP (Streit et al., 1989; Arvidsson et al., 1990; Haas et al., 1990; Dumoulin et al., 1991) are affected by peripheral axotomy. This study is the first to examine the motoneuron response of both endogenous substances to different types of motor nerve injury. The experimental design was to perform three types of nerve injuries at the same distance from the hypoglossal nucleus. This would result in three different patterns of nerve regeneration without deliberate mechanical impedance of axonal regrowth.

The main findings of this investigation consisted of differences in the early and late responses of ChAT and CGRP immunoreactivity to various types of injury. Dynamic variations in the **early response** of ChAT and CGRP to nerve injury were twofold: 1) upregulation of CGRP-IR preceded the down regulation of ChAT-IR and 2) the extent of regulation was related to the nature of the injury for ChAT, but not for CGRP. The chief differences in the **late response** to nerve injury included: 1) persistence of increased CGRP-IR was related to regeneration and

reinnervation of tongue musculature, while 2) ChAT-IR was a reversible process, regulated independently of reinnervation of tongue musculature.

#### Response of ChAT to Hypoglossal Nerve Injury

Changes in ChAT-IR have been evaluated after hypoglossal nerve crush (Armstrong et al., 1991) and nerve transection with proximal ligation (Lams et al., 1988; Armstrong et al., 1991). The present work is the first effort to evaluate quantitatively the responses of ChAT-IR to 3 different types of nerve injury and to provide correlative data on the time table for reinnervation of tongue musculature.

Qualitative differences in the down regulation of ChAT-IR at 3 days after nerve crush vs. nerve transection or resection agree with the findings of Armstrong et al. (1991) at 4 days after nerve crush vs. nerve transection with proximal ligation. However, our quantitative data reveal no significant differences in the reduction of ChAT-IR between nerve crush, transection or resection until 7 dpo. The dramatic reduction in ChAT-IR at 7 days after nerve transection or nerve resection, involving the majority of motoneuron somata and surrounding neuropil, is consistent with the extent of decrease in ChAT-IR at 6-9 days after nerve transection with ligation (Armstrong et al., 1991). Our results, like those of Armstrong et al. (1991), differ from the reported 50% reduction in ChAT-IR at 7 days after

hypoglossal nerve transection with ligation in adult Wistar rats (Lams et al., 1988). This discrepancy may reflect differences in the animal strain or nonuniformity in the methodology applied in ChAT labeling.

In the current study, qualitative and quantitative data demonstrate that ChAT-IR returned to normal levels by 20 days after nerve crush and was only moderately decreased at the same interval after nerve transection. By 50 days after nerve transection or resection, normal ChAT-IR levels had been attained. These results demonstrate that the progress in the return of normal neuronal levels of ChAT bears a temporal relationship to the nature of the injury. Furthermore, our findings derived from HRP retrograde labeling of hypoglossal motoneurons after tongue injections emphasize that the step-like reversibility of ChAT-IR was not dependent upon reinnervation of the lingual muscles. Equivalent retrograde labeling of the motoneuron population in both hypoglossal nuclei occurred in temporal correspondence with the return of ChAT-IR in nerve crush specimens. In contrast to these findings, only 24% and 1% of parent neurons were retrogradely labeled with HRP at 50 days after unilateral nerve transection or nerve resection respectively.

Why hypoglossal motoneurons, injured by axonal transection or resection, would resume synthesis of a transmitter-related enzyme without target contact is not clear. It is known that hypoglossal motoneurons survive

for periods up to a year after nerve transection (Snider and Thanedar, 1989) and that absence of ChAT-IR is not an absolute indicator of cell death of cholinergic neurons (Grafstein and McQuarrie, 1978; Lams et al., 1988). Furthermore, anterograde transport of ChAT in sciatic nerves was resumed by 29 days even after injury in which regeneration was prevented mechanically (Heiwall et al., 1979).

#### Response of CGRP to Hypoglossal Nerve Injury

The upregulation of CGRP-IR preceded the downregulation of ChAT-IR. The increase in CGRP-IR was obvious as early as 1 dpo and was of similiar magnitude for each type of nerve injury. This initial upregulation of peptide immunoreactivity corresponds temporally with that elicited in facial motoneurons after nerve transection (Streit et al., 1989). In contrast to these findings, no change in CGRP-IR was detected until two days after sciatic nerve transection in the midthigh of rats (Arvidsson et al., 1990). Recent evidence obtained from immunoassay of CGRP mRNA (Haas et al., 1990; Dumoulin et al., 1991) and in situ hybridization (Haas et al., 1990) demonstrated that upregulation of the peptide as early as 1 day in the facial motor nucleus is due to altered gene expression rather than decreased turnover rates of CGRP. Axonal lesions close to the cell body are followed by a more rapidly established perikaryal response (Lieberman, 1974). The delay in

increased CGRP-IR after sciatic nerve transection could therefore reflect a more distal location of the lesion. The current findings of dramatic CGRP-IR of equivalent intensity, as early as 1 day after each type of nerve lesion broaden the application of the idea that initial morphological features of the retrograde reaction are not influenced by the type of injury, whereas long term changes bear close relationship to the nature of the lesion.

In the present investigation, increased CGRP-IR was sustained at 7 and 20 days after nerve crush. At these same intervals after the other two injuries, however, the transient increase in peptide-IR declined to normal levels. Maximal decreases in CGRP-IR, occurring during the second postoperative week after nerve transection, have been reported (Streit et al., 1989; Arvidsson et al., 1990). A second peak in increased CGRP-mRNA levels has been demonstrated at the end of the third postoperative week in facial motoneurons (Haas et al., 1990), although correlative immunocytochemical evidence has not supported a biphasic pattern of peptide-IR (Streit et al., 1989).

Based on the current data, the prolonged upregulation of CGRP-IR after nerve crush vs. the transient increase in CGRP-IR after the other two injuries suggests a supportive role for CGRP in nerve regeneration. In this context, CGRP-IR in neurons could serve as a good predictor for effective outcome of axonal regrowth. Why the initial increase in peptide is presumably discontinued in the

injured nucleus of transected or resected specimens may be related to anterograde perturbations at the growing tip or retrograde reactions within the affected nucleus.

Studies on differences in the regenerative response to hypoglossal nerve crush, transection and ligation in rabbits (Frizell, 1982; Danielson et al., 1986), monitored by the front of transported radiolabeled materials, showed that initial axonal sprouting from the proximal segment of the injured nerve took place soon after each type of nerve injury. However, a 2-3 fold time delay in the transfer of materials from the injury site into the distal segment and the absence of a well-defined peak of radioactivity accounted for a less synchronous outgrowth pattern with variable regeneration rate in transected and ligated nerves. It is of interest that terminal swelling of the proximal stump, in the form of a neuroma, was found in transected and ligated nerves, but not in crushed nerves. Neuroma-like structures have been recognized by 7 dpo in transected, resected or ligated nerves in our laboratory (Borke, unpublished observation). CGRP has been suggested to be an anti-sprouting factor at the motor end plate (Tsujimoto and Kuno, 1988) and has been indicated to be involved in ectopic electrical activity at neuromas (Fried et al., 1989). The possibility may be raised that once functional contact with the target site is lost and axon terminals of the distal segment degenerate in the first postoperative week (Watson, 1974), prolonged accumulation of CGRP at an aberrant locus

such as a neuroma may influence the downregulation of CGRP in neurons. However, since HRP experiments in this investigation demonstrated retrograde labeling of 24% of hypoglossal neurons ipsilateral to the transection at 50 dpo, another increase in CGRP-IR should have been distinguished. Failure to detect a second increase in CGRP-IR could be due to the schedule of survival times used in these experiments. Examination of additional postoperative periods between 20-250 days may resolve this discrepancy.

An alternate possibility is that retrograde reactions that have a predilection to occur after transection and resection injuries may be implicated in the transient nature of the increased CGRP-IR. One such reaction that merits consideration is retraction of the dendritic tree of injured neurons. This retrograde reaction, which is initiated by 7 days after hypoglossal nerve transection (Sumner and Sutherland, 1973), consists chiefly of retraction and involution of dendrites coincident with or in response to partial deafferentation (Blinzinger & Kreutzberg, 1968; Sumner and Watson, 1971; Sumner and Sutherland, 1973; Sumner, 1975). This attenuation of dendrites is directly related to the severity of nerve injury. Reduction of the dendritic field is modest after nerve crush (Purves, 1975) but constitutes a characteristic feature of more severe injuries (Sumner and Watson, 1971; Sumner and Sutherland, 1973; Sumner, 1975, 1977). In addition to the reduction of the dendritic field from 7-14

days after nerve injury, dendritic profiles with inclusions can be identified. These profiles have been interpreted as signs of surface membrane activity (Sumner and Sutherland, 1973). Later, degenerating dendrites can also be seen, particularly in injuries where regeneration is impeded (Sumner and Sutherland, 1973; Sumner, 1977). The duration of the dendritic response is variable, since it persists until functional connections have been re-established with the target site. At this time re-expansion of the dendritic field takes place (Sumner and Watson, 1971).

In the current investigation, at 1-3 days after each type of nerve injury dendritic processes could be followed for considerable distances because they were replete with CGRP reaction product. The immunolabeling in dendrites was striking in contrast with CGRP-IR in uninjured and control hypoglossal nuclei, where it was chiefly limited to somata and proximal stems of dendrites. This marked CGRP-IR in dendrites is in agreement with that reported 1-6 dpo after facial nerve transection (Streit et al., 1989). This feature persisted to 20 days in our nerve crush specimens. However, it appeared reduced at 7 dpo in transected rats and was found only in some scattered neurons at 20-50 days after transection and 7-50 days after nerve resection. Thus the incidence and time frame of the apparent reduction in dendritic CGRP-IR coincide with that of dendritic retraction. In addition, the cross-sectional area of the affected nucleus was decreased at 20-50 days after nerve

transection or resection. Re-expansion of the hypoglossal nucleus has been reported after nerve transection (Sumner and Sutherland, 1973) but not after nerve injury in which regeneration was prevented (Sumner, 1977), emphasizing that temporary compression of the nucleus results chiefly from dendritic retraction rather than substantial dendritic degeneration.

One idea with regard to the current findings is that the decline in CGRP-IR at 7-50 days in transected or resected specimens resulted from a compensatory redistribution of CGRP into axonal processes in response to dendritic retraction. In this instance, the presumed discontinuance of CGRP-IR would be apparent, but in fact not real. An alternate explanation could be that dendritic retraction is associated directly or indirectly with the downregulation of CGRP synthesis. If this were so, the reduced levels of CGRP-IR reflect an authentic downregulation. Both of these hypotheses, if tenable, warrant further investigation.

Our qualitative data also conveyed the impression that CGRP-IR increased in the contralateral nucleus at 20-50 days after nerve transection or resection. Ultrastructural evidence of degeneration of dendrites in the contralateral nucleus after nerve transection has been reported (Sumner and Sutherland, 1973; Sumner, 1977). Since dendrites in the rat hypoglossal nucleus extend across the raphe to the contralateral nucleus (Wan et al., 1982), this degeneration

has been suggested to influence work hypertrophy in the uninjured, contralateral neurons. In this context, RNA synthesis has been shown to increase in the contralateral hypoglossal nucleus after transection (Watson, 1965). Although the side-to-side difference could represent a decrease in CGRP-IR below normal adult levels in the injured nucleus, this idea seems less likely because substantial neuronal cell death in the injured nucleus had not taken place. Whether increased CGRP-IR in the contralateral nucleus reflects a transneuronal influence from axonal injury should be more closely examined.

## CONCLUSION

The current study has served as an appropriate model to study the motoneuronal response of two endogenous substances, ChAT and CGRP, to nerve injuries of varying severity. Immunocytochemical results have shown that cellular levels of ChAT and CGRP respond in a diametric fashion, temporally and in regard to type of injury. Correlative data of reinnervation of tongue musculature were supplied by HRP labeling of hypoglossal motoneurons after nerve injury. Together these data provide a more complete picture of the role that a neurotransmitter-related protein, ChAT, and a putative trophic neuropeptide, CGRP, may play in hypoglossal nerve regeneration.

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